Microbial cytosine deaminases and their use in a new kind of cancer chemotherapy(Dissertation_全文)

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Contents

Abbreviations and definitions

A, absorbance; A_{xxx}, A at xxx nm
A, symbol of enzymic activity
5FC, 5-fluorocytosine
5FU, 5-fluorouracil
K_{av}, relative elution volume
L, litre(s); but ml for millilitre(s)
M, molar (unit of molarity)
N, normal (unit of normality)
n, number of samples
OD, optical density; OD_{xxx}, OD at xxx nm
r, correlation coefficient
SD, standard deviation
ssp., subspecies
t, symbol of time
U, units
UV, ultraviolet
±, plus/minus, used to express mean ± SD

CHAPTER 1

Introduction to this series of studies of microbial cytosine deaminases and their use in a new kind of cancer chemotherapy

In this chapter will be described the background and general features of this series of studies. The idea from which these studies arose originated from a model in nature, as is often the case in chemistry applied to living organisms: this origin was the antifungal activity of 5FC. This idea occurred to me when considering the role of cytosine deaminase present in certain fungi in the local chemotherapy with 5FC of diseases caused by these fungi. The antifungal activity is due to the cytosine deaminase of the fungus, which enzyme converts 5FC to 5FU. 5FC has no antitumoural or bacteriostatic activity, and little clinical toxicity. 5FU has antitumoural activity and a strong, broad-range antimicrobial spectrum, but is toxic. I wished to use 5FC in the treatment of cancer as the depot form of 5FU with the aid of microbial cytosine deaminase implanted locally.

1.1. *Metabolism of nucleic-acid-related compounds in organisms*

The microbial metabolism of nucleic-acid-related compounds has been studied at the Laboratory of Fermentation Chemistry at the University of Osaka Prefecture for many years. Chemistry in this field has developed rapidly, providing a base for industries having to do with pharmaceuticals, food, feeds, and other commodity chemicals. The industries in Japan concerned with fermentation technology lead the world.

Ribonucleotides are generally degraded to nucleosides through dephosphorylation by nucleotidases ① (figure 1-1a) or directly to bases through cleavage of nucleosidic bonds by pyrophosphorylases ② during the metabolism of nucleic-acid-related compounds. Nucleosides, including the ones formed as described above, are degraded to the corresponding purine and pyrimidine bases through removal of their ribose moieties with the participation of nucleosidases ③ or phosphorylases ④. Phosphotransferases (kinases) ⑤ convert nucleosides to nucleotides through phosphorylation

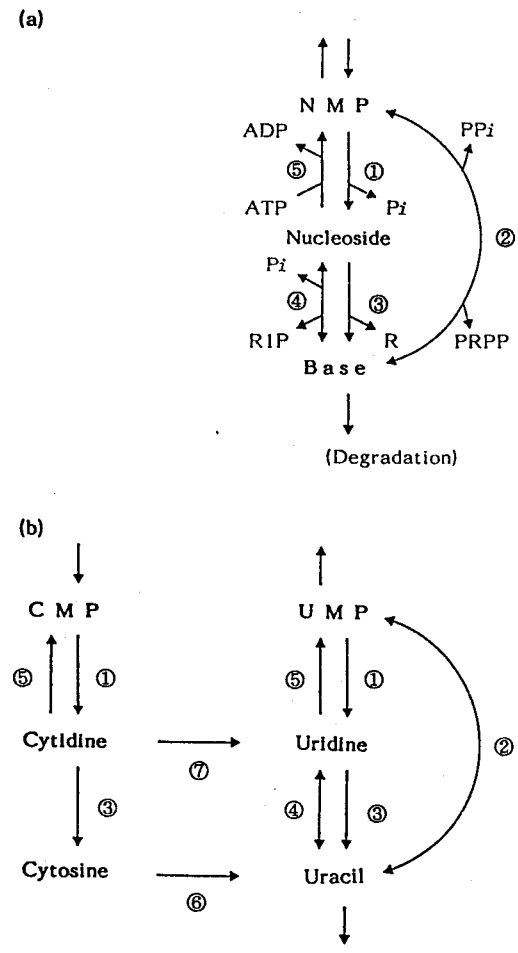

Figure 1-1. Metabolism of nucleosides.
Numbers for the enzymes in the figure are mentioned in the text.
(a) General.
(b) Pyrimidines.

and the reactions by the pyrophosphorylases ② and the phosphorylases ④ are reversible. So they are used as pathways for the salvage synthesis of nucleotides from bases. However, pyrophosphorylase ② and phosphorylase ④ for cytosine or cytidine are lacking (figure 1-1b). Cytosine and cytidine are first converted to uracil and uridine by cytosine deaminase ⑥ and cytidine deaminase ⑦, respectively (figure 1-1b). Cytosine, however, appears not to be metabolized in mammals [4,43], which have not a cytosine deaminase [21]. Uracil and uridine are resynthesized up to the level of nucleotides in the forms of uracil nucleotides (figure 1-1b), as usual, *via* the salvage synthetic pathway already described (figure 1-1a). Among the enzymes of these pathways, cytosine deaminase (cytosine aminohydrolase, EC 3.5.4.1; figure 1-2a) is unusual in that it is found only in microbes.

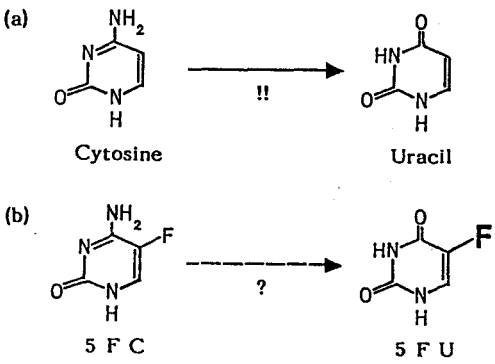

Figure 1-2. Cytosine deaminase.
(a) Action of the enzyme on cytosine.
(b) 5FC and 5FU, as possible substrate and product, respectively.

1.2. *Cytosine deaminase*

Cytosine deaminase was first found in bakers' yeast (*Saccharomyces cerevisiae*) [29] and *Escherichia coli* [30], and has been studied since then in many organisms in comparison with each other, together with the other enzymes related to nucleic acid metabolism (for review see [58,65,76]).

Large quantities of cytosine deaminases were partially purified from cultured *E. coli* and commercial compressed bakers' yeast and used in these studies. These cytosine deaminases were purified to high purity, and studied in detail. The *E. coli* enzyme was purified to homogeneity, and the properties of the purified enzyme were studied for the first time. The bakers' yeast enzyme was purified to an almost homogeneous state and then characterized. Its specific activity was several times higher than those reported before. A part of this report describes the protein and enzyme chemistry of these two enzymes.

1.3. *5FC as antifungal agent with participation of cytosine deaminase*

The motive for the beginning of these studies was that 5FC, a fluorinated analogue of cytosine [22,32] (figure 1-2b), has specific fungistatic properties *in vitro* [50] and *in vivo* [28, 50], and is of special utility, under the name flucytosine,

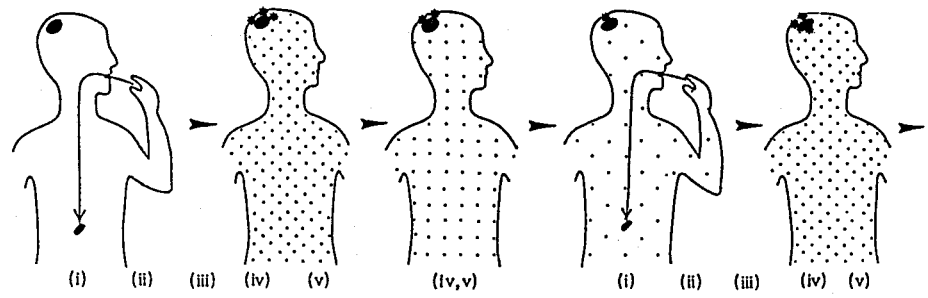

Figure 1-3. Mechanism of action of 5FC on fungal infections.

Fungal infection is in the brain (ellipse). A 5FC tablet is given orally, to be absorbed by the intestines. The 5FC concentration is shown by dots; 5FU formed shown by stars. Details are in the text.

etc., in deep refractory infections by yeasts and fungi [70] (for review see [6,33]). It has no antineoplastic activity [22], unlike 5FU (figure 1-2b), which is a fluorinated analogue of uracil, and which is currently used in chemotherapy for cancer [32]. 5FC and 5FU are incorporated into RNA, especially into tRNA, competitively with cytosine and uracil [27,39,43,60,61]. Current thinking about the mode of action of 5FU is that the main action is the interruption of DNA synthesis, which causes so-called thymineless death; that is, 5FU inhibits thymidylate synthetase as an antagonist of 2′-deoxyuridylate (dUMP) after the conversion of 5FU to 5-fluoro-2′-deoxyuridylate (5FdUMP) by the enzymes that normally act upon uracil [16,31]. 5FC acts in the same way after its conversion to 5FU in yeasts. Yeast growth is inhibited by 5FC [27]. The antifungal activity of 5FC has been attributed to the participation of the cytosine deaminase of the fungi themselves, which enzyme deaminates 5FC to 5FU [27,39] (figure 1-2b). 5FU is what actually acts on the fungi, and the mode of action of 5FC after its conversion into 5FU in yeast and fungus is the same as that of 5FU in these organisms and animal cells [20]. So the mechanism of action is as follows (figure 1-3). (i) The patient takes 5FC by mouth. (5FC is given periodically to maintain an effective concentration in the blood.) (ii) 5FC is absorbed into the blood from the intestinal tract. (iii) It circulates systemically and its concentration in the blood increases. (iv) It permeates the fungal cells in the diseased area. (v) The cytosine deaminase of the fungi themselves deaminates 5FC to 5FU. It is not until then that 5FC acts on the fungi.

1.4. *Side effects of 5FU*

5FU is an effective drug but has serious side effects [18],

and substitutes for it have been widely sought (for review see [62]). 5FC is one such substitute. It has mild side effects in the human body [52], in which 5FC is not metabolized, to judge from results of tracer experiments [43]. Differences in the action between 5FU and 5FC can be explained as follows. Cytosine deaminase is not present in animals, which is a more important factor in the lack of toxicity of 5FC in animals than the substrate specificity of the enzyme; that is, the ability to deaminate 5FC as well as cytosine. Tracer experiments showed that a small amount of 5FU appeared in rats after the oral administration of 5FC, the results of the action of bacteria resident in the intestine [43]. In humans, cytosine deaminase activity has not been found in any organ [21], but a small amount of 5FU has been detected in the blood, probably arising from intestinal microflora [21]. So 5FC is only partially metabolized in humans, and does not cause as serious side effects as 5FU. Ftorafur (*R,S*-1-(2-tetrahydrofuryl)-5-fluorouracil) [9] and 5-fluoro-5′-deoxyuridine (5FdUR) [10, 47], both derivatives of 5FU, are also among substitutes that have been suggested, but their active form may be 5FU after cleavage of the pseudonucleosidic bond [5, 72] and the ribonucleosidic bond [2], respectively. Thus, the problem of the overall clinical toxicity of 5FU may not have been totally eliminated. It has been proposed, however, that the cleavage of 5FdUR might be catalysed by uridine phosphorylase, which is abundant in tumour tissues in mice [37]. The same is true for humans for both drugs, as they are converted to 5FU by thymidine phosphorylase, which is abundant in human lung tumour tissues [45,46]. These drugs, then, cause no serious side effects because 5FU is generated at the local site of the disease.

1.5. *Drug-delivery system*

Most chemotherapeutic agents lack tumour specificity. Antimetabolic drugs generally inhibit the same aspect of metabolism in any class of cells; in any organism, or in any organ of the body. So, when used clinically, such drugs give similar side effects, to varying degrees. The intermittent administration of a drug by mouth or by injection generally provides a high level of the drug in the blood, and in the body, soon after the administration. The level then decreases with time until the next dose is given. This is called the peak-and-valley effect. To maintain the peaks and valleys within the proper range, the drug must be divided into smaller doses

and given frequently by mouth or through injections, or, if peaks and valleys are to be avoided as far as possible, given continually through intravenous infusion, which is uncomfortable and inconvenient. Such administration can be generally given only to hospitalized patients.

For chemotherapy, local generation of a drug near the diseased area would be ideal, as was mentioned for the cases of 5FU, ftorafur, and 5FdUR, with the participation of cytosine deaminase, uridine phosphorylase and thymidine phosphorylase. Almost all of the antimetabolites of nucleic-acid-related compounds inhibit DNA or RNA synthesis (or both) at various stages of the synthesis. 5FC [27] and 5FU [34], which are potent drugs, are included among these antimetabolites. They non-selectively injure nucleic acids in any organism, or in any organ of the body, and cause side effects. Their selective therapeutic effects are a reflection of the difference between normal and abnormal metabolism in sensitivity to the drug.

Local chemotherapy is an ideal treatment because the concentration of the drug is very low outside the diseased area, and very high in the diseased area. To solve the problem of side effects, improvements have been made in how a drug can be delivered more efficiently and more safely to the appropriate area by what is called a drug-delivery system. One approach is the active, site-directed delivery of a drug to the diseased area, which is called targeting. This kind of drug is popularly called a "magic bullet," and the therapy is called "missile" therapy. Another approach is the controlled release of the drug near the diseased area if possible, through dosage-form design, by implantation or injection in the body of a device or a so-called sustained- or slow-release drug in a shape of a pellet or a needle as shown in figure 1-4. Examples of implantable devices are pump systems such as the Ommaya reservoir [59] and "Infusaid" [7] shown in that figure.

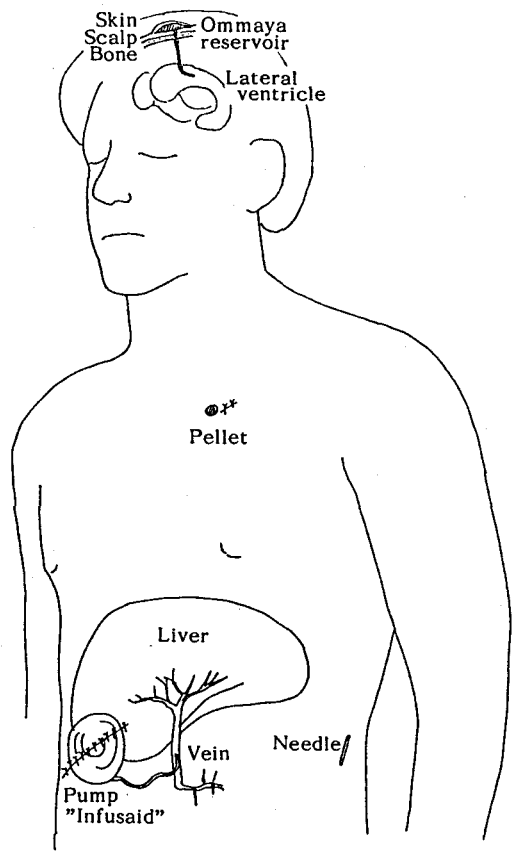

Figure 1-4. Drug-delivery devices implantable in a patient as shown.
Modified from [8].

1.6. *Approach to local chemotherapy of cancer with 5FC and cytosine deaminase*

My approach to a solution to the problem was a system for the controlled release of 5FU. The idea was to devise a way to use 5FC as a depot form of 5FU in the treatment of cancer, and the mode of action of 5FC in the proposed programme would be the same as for treatment of fungal diseases, as follows (figure 1-5). (Numbers, except for (o), correspond to

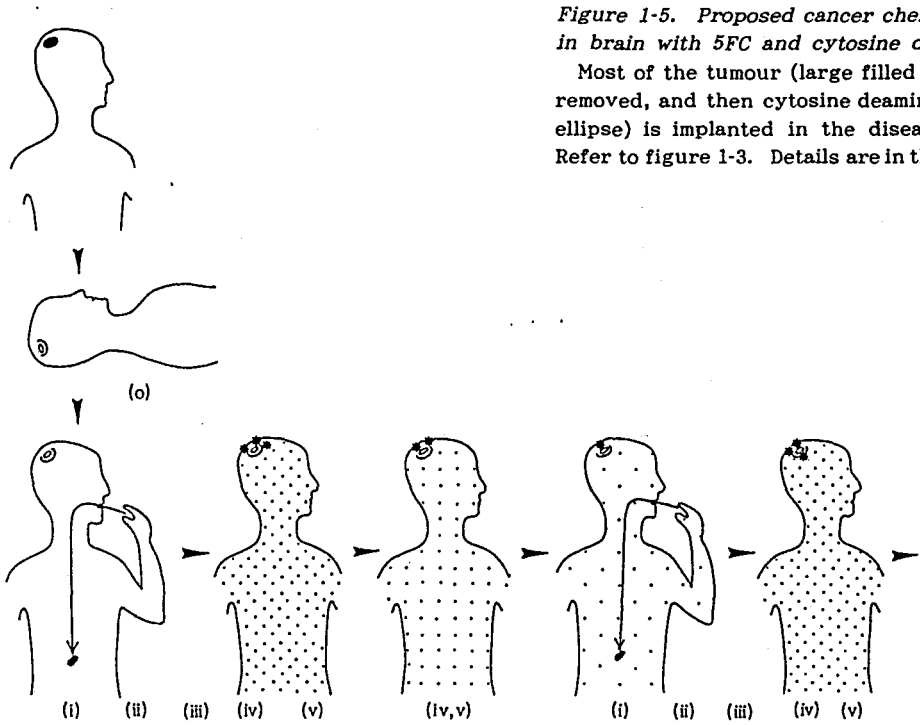

Figure 1-5. Proposed cancer chemotherapy in brain with 5FC and cytosine deaminase.
Most of the tumour (large filled ellipse) is removed, and then cytosine deaminase (open ellipse) is implanted in the diseased area. Refer to figure 1-3. Details are in the text.

those for fungal treatment described above and in figure 1-3.) (o) Cytosine deaminase (or its dosage form such as an enzyme capsule) would be injected or implanted near the tumour of a cancer patient, (i) 5FC would be given periodically to the patient by mouth, and (ii,iii) after it arrived at the enzyme capsule *via* the blood, (iv,v) it would be converted to 5FU at the local site by deamination by the action of the enzyme. This 5FU would then act on the tumour.

In therapy, the enzyme capsule could be implanted during the surgical removal of most of the tumour. This method might make it possible to use 5FC as postoperative therapy. Such therapy would be advantageous, especially in the brain as will be described below. This method would be also convenient as long-term care whether in the hospital or at home, because 5FC can be taken by mouth. This is not true of current modes of therapy, which involve the administration of direct antitumour agents alone or in some combination with other such agents or immunomodulators and physical treatments such as radiation and hyperthermia.

1.7. *Experimental brain tumour models*

Brain tumours are suitable models to evaluate the proposed therapy. These studies were done mainly with experimental brain tumours in rats. Resection of the tumour is effective for treatment or alleviation, as for other types of cancers, and is probably necessary in brain tumours. However, total resection is generally very harmful and must be avoided in the brain. So, infiltrating edges might remain behind that cannot be removed. Local drug treatment can be begun during surgery by placement of a drug or dosage system in the cavity made in the tumour bed.

Most agents do not readily pass the blood-brain barrier. This difficulty does not allow the maintenance of an effective concentration of the drug at the diseased site of the brain for a long enough period. Unnecessarily high or harmful concentrations of the drugs outside the brain may be brought about in an attempt to achieve effective levels of the drug in the brain. Fortunately, enough 5FC passes the blood-brain barrier to give sufficient levels even when given orally to treat fungal diseases.

1.8. *5FC-deaminating activity of cytosine deaminase*

It was found by chance that a cell extract of *E. coli* deaminated 5FC to 5FU as well as deaminating cytosine to uracil: this bacterium is known to have cytosine deaminase activity, as described above, and a cell extract of it happened to be on hand in the laboratory. This activity was also very stable to heat. Accordingly, I designed enzyme capsules containing cytosine deaminase, made with cellulose semipermeable membranes to avoid reaction of the enzyme protein with macromolecules in the body. *E. coli* was cultivated on a large scale, and cytosine deaminase was extracted from the culture. The enzyme was partially purified, and made into enzyme capsules. The capsules thus made were active *in vitro*, as the free enzyme solution was. Preliminary experiments with the enzyme capsules and with experimental brain tumours in rats were carried out in collaboration with neurosurgeons at Kansai Medical University, Moriguchi, Osaka.

1.9. *Active enzyme capsules of cytosine deaminases*

This study was started because of the encouraging results obtained in the above experiments [55]. First, a large quantity of cytosine deaminase was obtained from *E. coli* and bakers' yeast. Second, enzyme capsules was prepared with the *E. coli* enzyme, and these capsules were found to be active. Third, the yeast enzyme, which is very labile but is of a safer origin, was stabilized by immobilization in order to prepare improved enzyme capsules from this enzyme.

Studies done with my collaborators and an American firm involving animal experiments have been successful. Some of the results have been published [55~57], and the studies are still in progress [66].

1.10. "*New biotechnology*"

Thus, the idea of an enzyme capsule as an implantable drug-release device has been explored here. The idea suggests a new kind of application of enzymes.

It has been some time since the so-called "new technologies," including the field of "new biotechnologies," were first expected to contribute to the fields of the biosciences, such as medicine, pharmacy, biology, and biochemistry. Use of enzymes is an "old technology." Immobilization of enzymes is also relatively old; it was used here to increase the longevity of the life of the enzyme, but with "new materials" obtained from the use of "new technology."

CHAPTER 2

Cytosine deaminase from *Escherichia coli* with 5-fluorocytosine-deaminating activity and its possible use in cancer chemotherapy as implantable enzyme capsules [a]

A cell extract of *Escherichia coli* was found to have a cytoine deaminase that can stoichiometrically deaminate 5FC to 5FU. It was very stable to heat. The enzyme was partially purified and aseptically encapsulated in semipermeable cellulose tubes.

An enzyme capsule containing 0.91 U of activity was incubated in sterile saline at 37°C. After 16 days, it had some enzymic activity, and contained 0.31 U of activity.

Experiments were done with experimental brain tumours in rats. The other capsules, each containing 0.20 U of activity, were implanted under the skin. After one month, the capsules were removed, and found to contain 0.025 ± 0.011 U per capsule (biological half-life of 10 ± 2 days; mean $\pm$ SD, $n = 6$). These findings suggested that the capsules might be used for the chemotherapy of cancer.

2.1. INTRODUCTION

I wanted to devise a way to use 5FC as a depot form of 5FU according to the programme proposed for the treatment of cancer in Chapter 1. To start with, an enzyme that deaminated 5FC to 5FU was searched for. A cell extract of *Escherichia coli* that happened to be on hand was found to deaminate 5FC to 5FU. This activity was that of a cytosine deaminase that can stoichiometrically deaminate 5FC to 5FU as well as cytosine to uracil; the enzyme was very stable to heat. It was partially purified before use.

Enzyme capsules made with a semipermeable membrane that contained cytosine deaminase were designed; the semipermeable membrane was used to prevent reaction of the enzyme protein with macromolecules in the body.

A partially purified enzyme was aseptically encapsulated in cellulose dialysis tubes. Some *in vitro* and *in vivo* characteristics of these enzyme capsules are described here.

2.2. RESULTS

2.2.1. In vitro *conversion of 5FC into 5FU*

Spectrophotometric confirmation. First I checked whether

E. coli K-12 IFO 3301 deaminated 5FC to 5FU, using a cell extract that happened to be on hand. The reaction mixture contained 0.75 ml of the cell extract, 1 μmol of 5FC, and 0.2 mmol of potassium phosphate buffer (pH 7.0) in 1 ml, and was incubated at 37℃. Portions were withdrawn at 0, 12, and 24 hr, diluted 50 times with 0.1 N HCl, and centrifuged at 20,000 $\times g$ for 15 min; UV absorption spectra of the supernatants were taken against the corresponding diluted blanks (the reaction mixture without 5FC). The spectrum at 0 hr was similar to that of the authentic 5FC solution, that at 24 hr was similar to that of the 5FU solution, and that at 12 hr was midway between, with the absorption maximum shifted towards that of 5FU (data not shown). This suggested that most of the 5FC was converted into 5FU within 24 hr, and that the activity was very stable to heat under the above conditions.

Stoichiometry. A cell extract of *E. coli* was incubated with 5FC at 37℃ and pH 7.4, and at intervals the incubation mixture was analysed for 5FC and 5FU by paper chromatography (figure 2-1a). The assay results suggested that conversion was stoichiometric. The same cell extract catalysed the 1 : 1 conversion of cytosine into uracil as well (figure 2-1b).

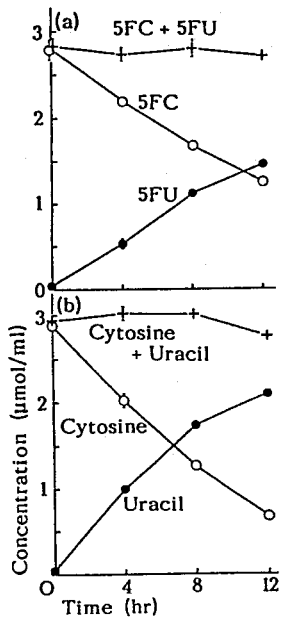

Figure 2-1. Conversion of (a) *5FC to 5FU and* (b) *cytosine to uracil with a cell extract of* E. coli.

The reaction mixture contained, in 1 ml, 0.6 ml of the cell extract (2.4 mg as protein), 3 μmol of (a) 5FC or (b) cytosine, and 0.1 mmol of potassium phosphate buffer (pH 7.4), and was incubated at 37℃. At 4-hr intervals, portions were withdrawn from the reaction mixture, heated to stop the reaction, and chromatographed on paper to assay (a) 5FC and 5FU, and (b) cytosine and uracil. The mean of three independent determinations was calculated; vertical bars show SD larger than 0.06 mM. Smaller SD values do not appear, being hidden behind the symbols.

2.2.2. *Enzyme capsules*

Encapsulation of the enzyme solution might prevent allergic reactions effectively and preserve enzyme activity by separation of the enzyme protein from the surroundings. The enzyme was partially purified from cell extracts of *E. coli* grown on peptone and citrate by processes to be described in Chapter 3, and filtered through a membrane filter to sterilize it. The sterile solution was pipetted into autoclaved cellulose dialysis tubes already tightly tied on one end with silk thread. Then the other end was tied. The ends were further sealed with surgical cement. Sometimes empty capsules made of cellulose dialysis tubes and silicone rubber stoppers were used; then the enzyme solution was injected (figure 2-2). These enzyme capsules were made of a membrane with a molecular-weight cutoff of 1,000, so they should allow almost free permeation of 5FC and 5FU, but trap the enzyme protein inside, protecting it from attack from the outside.

2.2.3. *Intact enzymic activity of capsules*

An enzyme capsule containing 0.91 U of activity was

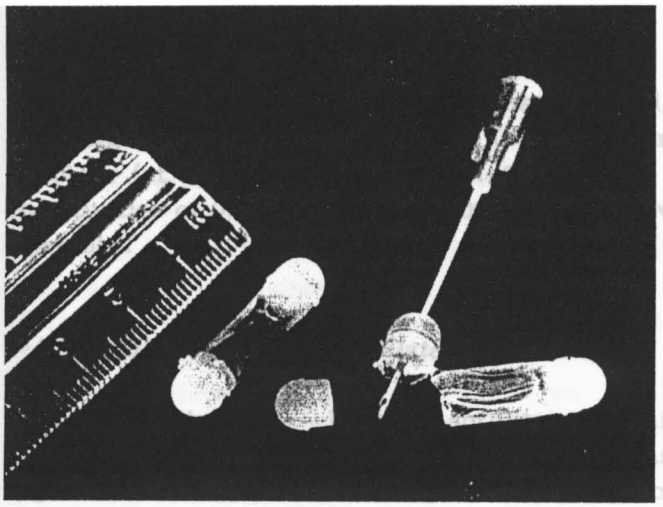

Figure 2-2. Enzyme capsules.

Round-ended enzyme capsules are shown (almost actual size). Cellulose dialysis tubing was sealed with silicone-rubber stoppers made to order by die casting. The tubes and stoppers were sterilized by autoclaving, and the following steps were carried out on a clean bench. The inside of the edges of both tube ends was wetted with surgical cement, and the stoppers were immediately inserted. After the cement set, the ends were bound with silk thread. Sterile enzyme solution was then injected into the capsule through an injector needle piercing the stopper, removing the air inside through the same needle when necessary. The capsules thus made were stored in buffered saline in a refrigerator.

incubated for 16 days at 37℃ in several changes of sterile phosphate-buffered saline. Then, it was allowed to react at 37℃ with cytosine in phosphate-buffered saline in a semi-continuous way (figure 2-3). Periodic replacement of portions of the reaction mixture outside the capsule made it possible to maintain semi-stationary concentrations of cytosine and uracil (figure 2-3a,b), and, therefore, gave a semi-constant rate of the conversion of cytosine into uracil (figure 2-3aa,bb). When the mean concentration of cytosine was 0.3 mM, the rate of conversion was 0.2 μ mol/hr (figure

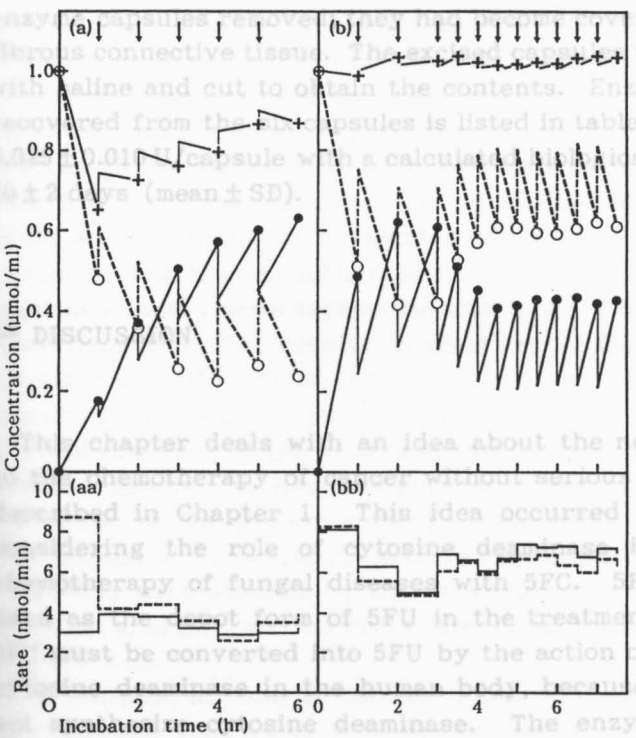

Figure 2-3. Deamination of cytosine by an intact cytosine deaminase capsule.

The enzyme capsule described in the text was wiped with paper, put in a test tube containing 1.0 ml of 1 mM cytosine in phosphate-buffered saline, and incubated at 37℃. At the times indicated by arrows, 0.25-ml volumes of the solution outside the capsule were changed for fresh cytosine solution. After this experiment (a), the enzyme capsule was immersed in several changes of a few millilitres of cytosine solution for several hours in a refrigerator. The second experiment (b) was carried out in the same way, except that the replacements of the reaction mixture were 0.50 ml each time. The solutions removed from the reaction mixtures were assayed for cytosine and uracil concentrations by spectrophotometry.

Calculations gave (a,b) the initial concentrations of cytosine and uracil in the next incubations, and, therefore, (aa,bb) the rates of conversion (*i.e.*, the decrease in the amount of cytosine and the appearance of uracil during the incubations, expressed by horizontal bars in the figure).

Symbols represent single measurements. In (a) and (b), ○ and ----- are cytosine; ● and —— are uracil; and —— are the sum of cytosine and uracil concentrations. In (aa) and (bb), ---- is cytosine and ——— is uracil.

2-3a); when it was 0.5 mM, the rate was 0.3 μmol/hr (at 2～3 hr in figure 2-3b); and when it was 0.7 mM, the rate was 0.4 μmol/hr (at 5～7 hr in figure 2-3b). These rates correspond to activities of 0.003, 0.005, and 0.007 U, respectively (figure 2-3aa,bb).

2.2.4. *Stability of the encapsulated enzyme*

In vitro *stability.* Enzyme activity remaining in the capsule after long-term incubation at 37℃ was examined in the capsules used in the above experiment. The capsule was cut over a graduated cylinder with scissors, and the contents were collected with small amounts of phosphate-buffered saline. The total activity recovered was estimated to be 0.31 U, and the rate of decrease of the enzyme activity of the capsule was calculated to have a biological half-life of 10.3 days.

In vivo *stability.* Stability of the activity was also examined in rats with experimental brain tumours borne under the skin. One enzyme capsule containing 0.20 U of activity was implanted in the tumour bed of each of six rats through skin incisions near the tumours. The rats thereafter received daily intraperitoneal doses of 150 mg of 5FC/kg. After 30 days of therapy, the tumours were incised and the enzyme capsules removed; they had become covered by thick fibrous connective tissue. The excised capsules were washed with saline and cut to obtain the contents. Enzyme activity recovered from the six capsules is listed in table 2-1 and was 0.025 ± 0.010 U/capsule with a calculated biological half-life of 10 ± 2 days (mean ± SD).

Table 2-1. Cytosine deaminase activity remaining in capsules after 30 days of implantation in rats.

Six enzyme capsules each containing 0.20 U of activity were implanted in rats. Details are given in the text.

Capsule no.	Residual activity		Half-life (days)
	(U)	(%)	
1	0.035	18	12
2	0.030	15	11
3	0.028	14	10
4	0.008	4	6
5	0.018	9	9
6	0.035	18	12

2.3. DISCUSSION

This chapter deals with an idea about the new approach to the chemotherapy of cancer without serious side effects described in Chapter 1. This idea occurred to me when considering the role of cytosine deaminase in the local chemotherapy of fungal diseases with 5FC. 5FC might be used as the depot form of 5FU in the treatment of cancer. 5FC must be converted into 5FU by the action of exogenous cytosine deaminase in the human body, because humans do not synthesize cytosine deaminase. The enzyme must be immobilized and placed at the site where it is to take effect.

In addition to the pathogenic fungi described before, some microorganisms have cytosine deaminase activity [74]. This enzyme, when obtained from bakers' yeast or *Pseudomonas aureofaciens*, is unstable to heat, and when from *Salmonella typhimurium*, is thermostable [36,64,76]. I found that this enzyme from *E. coli* would deaminate 5FC to 5FU and was very stable, as shown here, so I used it.

The experiments here were carried out to find whether requirements for the proposed cancer chemotherapy were fulfilled. First, 5FC must be delivered to the diseased area: this should occur, as it does in the chemotherapy of fungal diseases with 5FC. Second, the enzyme capsule must deaminate 5FC to form 5FU *in situ*: this was found here to be true (figure 2-1). Third, the enzyme must have long-term stability in the body. The biological half-life of activity in the implanted capsules was calculated to be about 10 days (mean for the capsule described in the text and the six capsules of table 2-1). In fact, these three requirements have been found to be fulfilled at once in a later *in vivo* experiment, which was done in collaboration with the neurosurgeons. The results, which were described elsewhere [57] and summarized in figure 2-4, show that, first, 5FC reaches the tumour (because the 5FC concentrations in the tumour and the serum were similar); second, the intact enzyme capsule is active (from the fact that 5FU appeared in the effective concentrations in the tumour), and that, third, the enzymic activity was stable (because the above capsule was active after 14 days of implantation in the animals). The stability of the cytosine deaminase activity demonstrated in the above two experiments is not enough for actual therapy. However, this problem can probably be overcome, because the enzyme preparations used here were only partially purified, and had not been stabilized by immobilization techniques such as entrapping or covalent coupling.

Concerning the chemotherapeutic activity of the enzyme capsules, local chemotherapeutic studies have been reported elsewhere [57]. The results, summarized in figure 2-5, show that the growth rate of the subcutaneous tumours in the control group was 9 days as expressed as the time required for the doubling of the volume of tumour, and 20 days in the treated group. It means significant, but not sufficient, reduction of the tumour growth. Concerning the safety of the capsules in therapy, passive cutaneous anaphylaxis reactions were studied by the neurosurgeons using guinea pigs; no allergic reaction in the host arose from the capsules (figure 2-6).

In therapy, an enzyme capsule could be implanted during the surgical removal of most of the tumour. This method

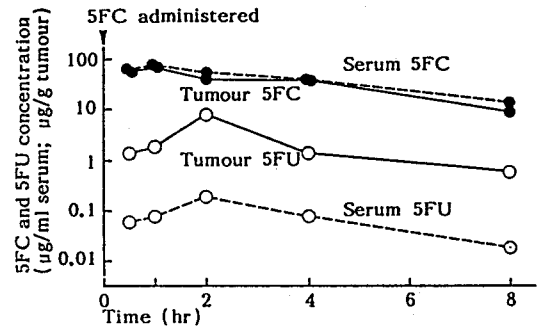

Figure 2-4. Concentration of 5FC and 5FU after administration of 5FC to rats implanted with cytosine deaminase capsules.

A cytosine deaminase capsule containing 1 U of activity was implanted in the bed of experimental brain tumours (about 2 cm^3) of ten rats. After 14 days, 150 mg of 5FC per kg was injected intraperitoneally. The rats were then killed at different times and blood and tumour tissues were sampled. Sera were separated and the tissues were homogenized. The 5FC and 5FU concentrations of the samples were measured by microbiological assay.

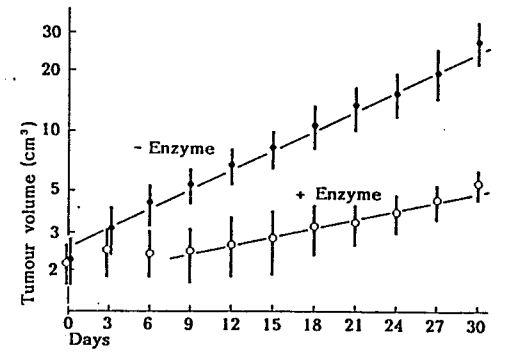

Figure 2-5. Inhibition of growth of tumours of rats implanted with enzyme capsules and treated with 5FC.

Modified from [57]. A cytosine deaminase capsule was implanted in tumour-bearing rats (○; $n=10$). The control was a capsule containing buffered saline (●; $n=10$). Starting on the fourth day, the animals were given 150 mg of 5FC per kg daily. The tumour was measured from outside the skin; tumour volume was calculated as $^1/_2 \times$ length $\times$ width $\times$ height (cm^3). With treatment, the doubling time for the weight of tumour was calculated to be 20 days, but 9 days for the control. On the 30th day, the rats were killed, and the tumours were extirpated and weighed. The weight was 8.8 ± 2.2 g for the rats implanted with an capsule, and 34.5 ± 8.0 g for the control (slightly smaller than calculated as above).

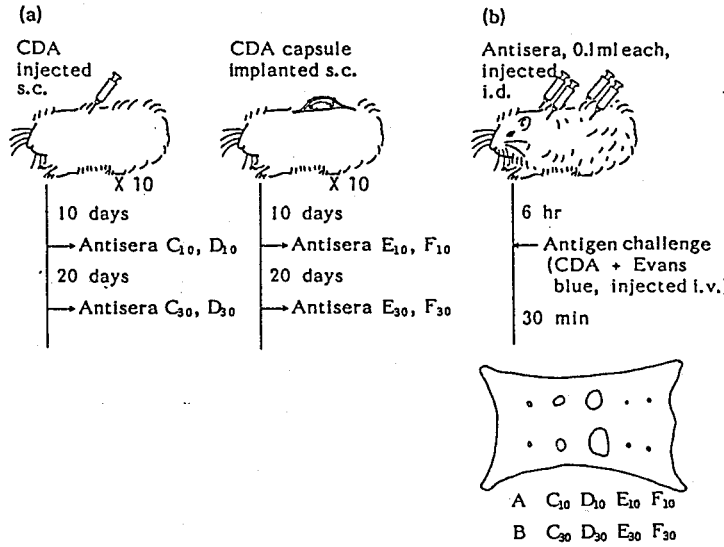

Figure 2-6. Polyclonal antibody reactions. Cited from [57].

(a) *Sensitization and antisera.* Antisera were produced in animals C and D given a subcutaneous injection of cytosine deaminase solution or in animals E and F implanted with an enzyme capsule. Antisera were obtained 10 and again 30 days after the sensitization. (b) *Passive cutaneous anaphylaxis test.* The antisera, together with saline (A) and the control serum (B), were injected intradermally into an animal's back, and 6 hr later, the antigen in the form of an enzyme solution containing Evans blue was given intravenously. Thirty minutes after the challenge, the animal was killed and skinned, and the antibody reactions were read on the inner side of the skin.

might make it possible to resect a tumour, leaving its infiltrating edges behind as necessary, and then to use 5FC as postoperative therapy. Such therapy would be advantageous, especially in the brain, where total resection of the diseased part is difficult. This method would be also convenient as long-term postoperative care whether in the hospital or at home, because 5FC can be taken by mouth. This is not true of current modes of therapy with direct antitumour agents, radiation, *etc.*

2.4. EXPERIMENTAL

Unless otherwise noted, all experiments were done in the cold and the buffer used was potassium phosphate, pH 7.0, called "the buffer" and was usually used at the concentration of 50 mM. The phosphate-buffered saline used was 0.82% NaCl solution containing 10 mM potassium phosphate buffer (pH 7.4).

2.4.1. *Materials*

Chemicals. All chemicals unless otherwise noted were obtained from Wako Pure Chemical Industries (Osaka), and were of certified reagent grade.

Cytosine and uracil were products of the Yamasa Shoyu Co. (Choshi, Chiba). 5FC was from Nippon Roche KK (Tokyo) and 5FU from Kyowa Hakko Kogyo (Tokyo). The peptone (Polypepton) and the yeast extract (practical grade product) were from the Nihon Pharmaceutical Co. (Tokyo). The meat extract was Ehrlich meat extract produced by the Kyokutō Pharmaceutical Industrial Co. (Tokyo). Antifoam Silicone KM-70 was the product of the Shin-Etsu Chemical Co. (Tokyo).

Other materials. The filter paper was no. 52 of the Toyo Roshi Kaisha (Tokyo). The semipermeable cellulose membrane used to prepare the enzyme capsules was Spectra/Por 6 dialysis tubing (Spectrum Medical Industries, Los Angeles). It is made from regenerated cellulose, and has a molecular-weight cutoff of 1,000, and is 8 mm wide when flattened out. The membrane filters used in the aseptic procedure were 0.2-μm Nuclepore membranes fitted in 13-mm pop-top holders (Nuclepore Corp., Pleasanton, Cal.). The surgical cement used, Aron Alpha A (Sankyo Co., Tokyo), was a cyanoacrylate.

Instruments. The ultrasonic oscillator operated at 19.5 kHz (type 4280; Kaijo Denki Co., Tokyo). The wavelength of the UV lamp was 254 nm (hand-held type; Manaslu Chemical Industry, Tokyo). The recording spectrophotometer was of the double-beam type (model 124; Hitachi Ltd., Tokyo).

2.4.2. *Culture and cell extract*

E. coli K-12 IFO 3301 was cultivated with a 1% inoculum at 30℃ for 1 day in flasks on a shaker, in a growth medium composed of 3% peptone, 1% glycerol, 1% KH_2PO_4, 0.6% yeast extract, 0.3% NaCl, and 0.01% $MgCl_2$ in tap water, and adjusted to pH 7.0 with NaOH solution [63]. The cells were harvested by centrifugation, washed in a 10-fold volume of the buffer, suspended in the same volume of the buffer, disrupted thoroughly with the ultrasonic oscillator, centrifuged to remove cell debris, and dialysed overnight against the buffer. The resulting solution is referred to as the cell extract.

2.4.3. *Paper chromatography*

Cytosine and uracil from the reaction mixtures were separated by paper chromatography with *n*-butanol/acetic acid/water (4 : 1 : 1, by vol.) by the ascending method. Then, 50-μl samples were applied in 2-cm lines with spaces of 2 cm between them along the starting line of no. 52 filter paper. Reaction mixtures without substrates provided blanks. After overnight development and drying, the chromatograms were examined under a UV lamp. The UV-absorbing areas were outlined with a pencil and, together with blanks, were excised with as little paper as possible so that they were identical in size, shape, and position. They were eluted with 5-ml volumes of 0.1 N HCl. The bases were identified and estimated from the R_F values and from the UV absorption spectra of the eluates against the corresponding blank eluates.

5FC and 5FU were separated in the same way. In addition, for better separation, chromatograms of reaction mixtures containing these compounds were developed again. The filter paper was cut 2 cm below the lowest UV-absorbing area, the lower portion was discarded, and solvent was sprayed on the rest of the sheet from the lower edge to the uppermost UV-absorbing area. Then development was repeated in the same direction as before.

2.4.4. *Enzyme and protein assays*

The activity of cytosine deaminase was assayed with cytosine as the substrate by a discontinuous method with differential spectrophotometry [63] modified from continuous method [36,40] based on the absorption of the substrate, cytosine, to the product, uracil, at the wavelength of 280 nm, where the differential molecular extinction coefficient is 8,230 $M^{-1}cm^{-1}$ in acidic solution [35]. The assay system contained 3 μmol of cytosine, and 200 μmol of potassium phosphate buffer, pH 8.0; the enzyme solution was assayed in a final volume of 1.0 ml. The mixture was incubated at 37℃ for 5 min and the reaction was started by the addition of cytosine solution. A portion was immediately withdrawn for the zero-time assay. After further incubation, usually for 30 min, the reaction was stopped by the addition of 24 volumes of 0.1 N HCl to the portion withdrawn at the time. Any precipitate that formed was removed by centrifugation, and the supernatants were analysed. Decreases in the cytosine concentration and increases in the uracil concentration from time zero to the time of the sampling was estimated by the decrease in OD_{280}. One unit (U) of activity is defined as the amount of the enzyme that catalyses the deamination of 1 μmol of cytosine per minute under the above conditions.

Protein was assayed by the Folin method with ovalbumin as the standard [49].

2.4.5. *Spectrophotometric determination of cytosine and uracil*

Cytosine and uracil, when measured at the same time, were measured with the assumption that the reaction mixtures contained only cytosine and uracil in buffered saline, and that the absorption spectrum showed the sum of those substantances. Samples and buffered saline (as blank) were diluted in 0.1 N HCl, and UV absorption spectra were taken. Calculations were made from spectral data read at 250 and 280 nm, where the differential absorption of uracil against cytosine is maximum and minimum, respectively, and where the molecular extinction coefficient of cytosine is 3,030 and 9,720 $M^{-1}cm^{-1}$, that of uracil being 6,810 and 1,490 [35]. The concentrations of cytosine and uracil were estimated by the solving of two simultaneous binomial equations with use of the absorbance at these wavelengths.

2.4.6. *Animals and experimental brain tumour*

Experimental subcutaneous tumours (EA-285 glioma) [81] were grown in the right half of the backs of male Fisher S 344F DUCRJ rats [57].

CHAPTER 3

Visual agar-plate assay for deaminases with use of a pH indicator [b]

I devised a simple, rapid method for the detection and assay of cytosine deaminase. The method makes use of paper disks of the enzyme, agar plates containing cytosine, and a pH indicator. The time required is short (6 hr or so) regardless of the scale of the assay (*e.g.*, several samples in a Petri dish or a few dozen samples in a rectangular tray). First, a method was developed for the assay of deaminases with the use of adenosine deaminase as a model, because this enzyme is commercially available. This method was adapted from a method reported elsewhere for zymoautography of adenosine deaminase in elecrophoresis gels and its modification with paper disks and agar gels contained in a multiwell micro-culture tray.

Second, this method was used with cytosine deaminase, the subject of this series of studies.

Third, the disk-plate method, with use of paper disks and agar plates, was used to detect and estimate amounts of specific inhibitors of adenosine deaminase.

This proposed method with use of a pH indicator is advantageous when used to detect activity in a large number of samples such as fractions from column chromatography of deaminases or their inhibitors. After several hours of practice, activity can be detected at a glance.

3.1. INTRODUCTION

An easy method to assay deaminases was first devised before the large-scale purification of cytosine deaminase was started. A method to detect adenosine deaminase activity in gels for electrophoresis has been reported that involves zymoautography in agar plates containing adenosine and a pH indicator [12]. A modification of this method has also been reported, which method is used for screening for adenosine deaminase deficiency in infants with paper disks of spotted blood instead of electrophoresis gels and with agar gels filling the wells of multiwell micro-culture trays instead of flat trays [53].

Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) was suitable for use in the experiment because this enzyme from bovine internal organs is commercially available. Usually, adenosine deaminase is readily assayed by both continuous and discontinuous spectrophotometry based on the differential absorption at 265 nm of the substrate, adenosine, to the product, inosine [40] (for review see [54]). This method has been used to assay cytosine deaminase [36]. Measurement of the ammonia liberated in the reaction of adenosine deaminase is also employed in the assays of the enzyme. Conway's micro-diffusion method [3] is the usual one for the measurement of ammonia, and was adapted to assay of adenosine deaminase in discontinuous assays [12]. However, the use of pH indicators enables detection of deaminase activity without termination of the reaction,

because ammonia changes colour continuously and gradually, towards its alkaline colouring as it is liberated during deamination.

Next, a disk-plate method for the assay of adenosine deaminase was developed and applied to the assay of cytosine deaminase, the subject of this series of studies. In addition, the above zymoautography of the enzyme was adapted to detect specific inhibitors of adenosine deaminase, and the disk-plate assay was adapted to assay them. These two techniques, which correspond to the bioautography and disk-plate microbioassay of antibiotics, respectively, are discussed here.

3.2. RESULTS

3.2.1. *Assay of adenosine deaminase*

The strategy to develop a visual assay of deaminases was put into practice with the adenosine deaminase assay as a starter.

Paper disks were wetted with solutions of adenosine deaminase at concentrations of 1, 10, and 25 μg/ml and placed on agar plates about 2 mm thick, that contained 1% agar, 0.01% phenol red (phenolsulphonphthalein), and a combination of 3, 10, or 30 mM adenosine and 1, 3, or 10 mM potassium phosphate buffer, pH 5.9, after which incubation was conducted at 37℃. At times during the incubation, the plates were observed for changes in the colour of the disks and the surrounding agar from yellow or pale orange to purplish red. The zones of changed colour were measured. At 2 hr, changes in colour were visible in most plates. Figure 3-1 shows the plates after 4 hr of incubation. The zones of colour change were blurry, but the diameter of the zone *versus* the logarithm of the concentration of the enzyme in solution was correlated; when plotted, these values for each plate were approximately linear (data not shown).

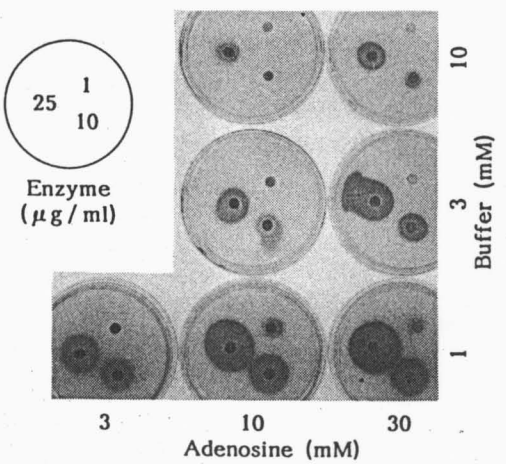

Figure 3-1. Adenosine deaminase assay.
Agar used for assays in the Petri dishes contained adenosine and buffer at the concentrations indicated. Three paper disks for each dish were wetted with enzyme solution at the concentrations of 1, 10, or 25 μg/ml and placed as shown.

3.2.2. *Assay of cytosine deaminase*

This technique was adapted to the assay of cytosine deaminase. Cytosine replaced adenosine in the above assay mixture. A typical example of the technique is as follows. A partially purified fraction, containing 63 U of activity and 10 g of protein, was chromatographed on a column (5ϕ × 27 cm)

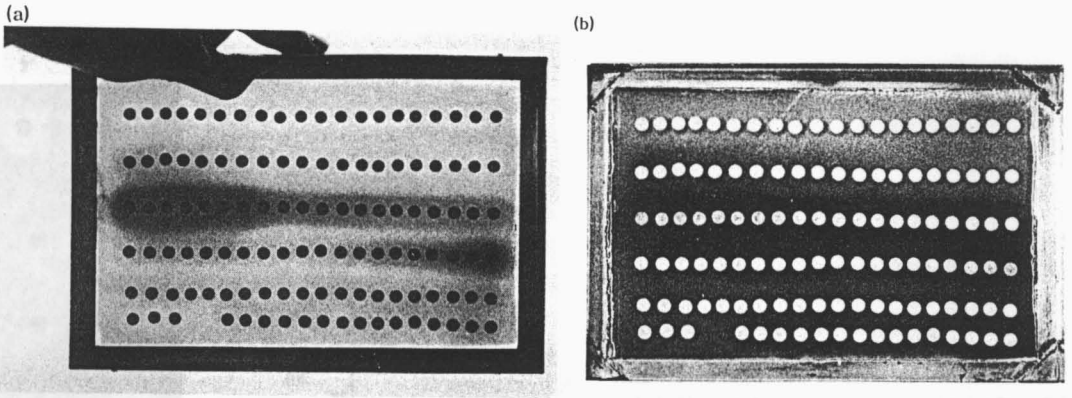

Figure 3-2. Visual detection of cytosine-deaminase-active fractions in column chromatography.

The plate was photographed after 6 hr of incubation.

(a) On a light box.

(b) Under lamps and on a white background. The paper disks of the active fractions turned purple (dark in the photograph); the others remained yellow (light in the photograph).

of DEAE-cellulose. Elution was with a linear gradient of KCl from 0 to 0.5 M in 2.0 L of the buffer, and 20-ml fractions were collected. Paper disks of the fractions were placed on an agar plate, about 2 mm thick that contained 5 mM cytosine, 1 mM potassium phosphate buffer, pH 5.9, and 0.01% phenol red, and the plate was incubated at 37℃. After a few hours, active fractions could readily be detected as belts of purplish red on a yellowish orange background (figure 3-2).

3.2.3. *Assay of adenosine deaminase inhibitor*

The method for adenosine deaminase was applied to the assay of the specific inhibitors of adenosine deaminase. The assay was by the disk-plate technique, as follows. The agar contained 6 μg of adenosine deaminase, 0.5 mmol of adenosine, 0.25 mmol of potassium phosphate buffer, pH 5.9, 20 mg of phenol red, and 1 g of agar in 100 ml of deionized water; the agar was formed into a plate about 2 mm thick. Paper disks were wetted with solutions of guanosine, 6-chloroguanosine, coformycin, and the J-518S substance, which is a strong specific inhibitor discovered in the laboratory (unpublished), at various concentrations. Incubation was at 37℃. After a few hours, coformycin and the J-518S substance, which are specific inhibitors, caused zones of yellow colour to remain around the edges of the paper disks, behind the purplish red that had appeared (seen as whitish and dark areas, respectively, in figure 3-3a). Guanosine and 6-chloroguanosine, which are competitive inhibitors, did not. The inhibitory zones did not change size further with the passage of time. Figure 3-3a shows a plate after 6 hr of incubation.

Calibration curve. The borders of the inhibitory zones were unclear, but the approximate diameters of the zones could be read. Zone readings for coformycin were made from the same plate as above after about 6 hr of incubation with

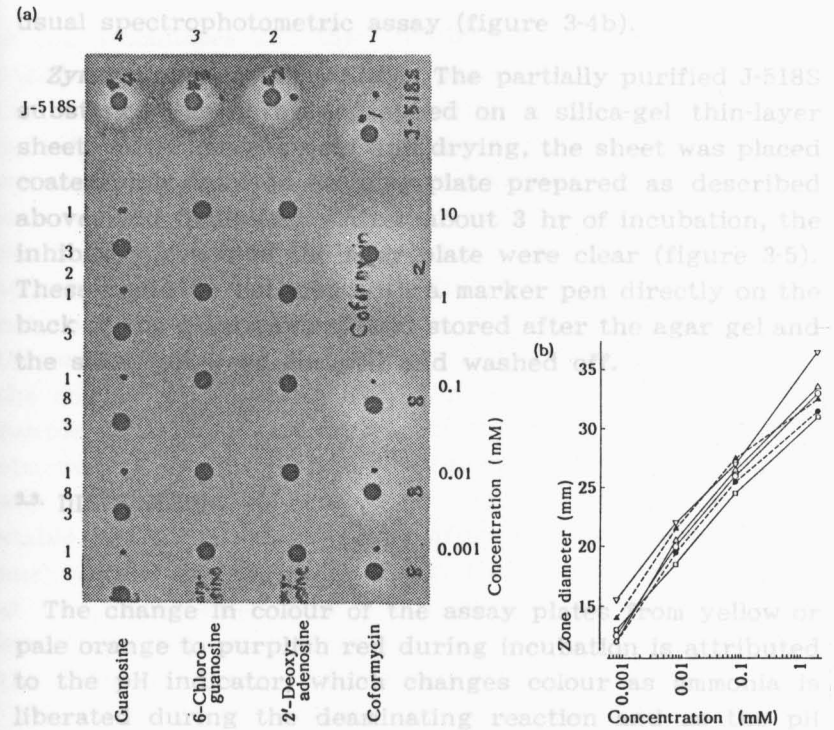

Figure 3-3. Plate assay of some classes of inhibitors against adenosine deaminase.

(a) The plate was photographed after 6 hr of incubation. The concentrations of the four inhibitors are indicated by their vertical position in the plate, for which digits and positional notations are marked at the left and right side, respectively. Numberings of J-518S disks are the sample numbers, which are fractions from chromatography.

(b) Zone readings for the coformycin disks were made in the same plate at about the same time as above. The relationship between zone size and the concentration of coformycin was semilogarithmic.

use of vernier calipers by six untrained volunteers who did not know what results were expected, and plotted against logarithms of the concentrations of coformycin (figure 3-3b). Each plot was approximately linear and parallel to the others, although the plots lay within a wide range because of the unclear borders (not because of differences in time).

Column chromatography of J-518S. The partially purified J-518S substance was chromatographed on a silicic-acid column, and serial samples from the fractions collected were assayed for inhibitory activity in paper disks on the agar plate (figure 3-4). The active fractions (no. 43 and later) are detected as yellow zones. Sizes of the inhibitory zones corresponded fairly well to the activity estimated by the

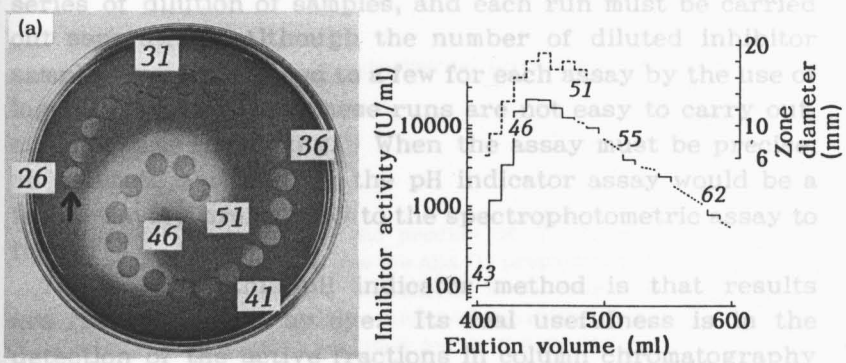

Figure 3-4. Detection of inhibitor fractions in column chromatography.

A test sample was chromatographed on a silicic-acid column.

(a) Paper disks of the fractions were placed on the assay agar in a Petri dish and incubated. Numbers in the figure are the fraction numbers.

(b) The active fractions thus detected and adjacent fractions were then assayed spectrophotometrically for inhibition in the usual way.

——, Activity by usual assay; – – –, zone reading in plate assay.

usual spectrophotometric assay (figure 3-4b).

Zymoautography of J-518S. The partially purified J-518S substance was chromatographed on a silica-gel thin-layer sheet. After development and drying, the sheet was placed coated side down on an agar plate prepared as described above, and incubated. After about 3 hr of incubation, the inhibitory zones in the agar plate were clear (figure 3-5). These could be outlined with a marker pen directly on the back of the plastic sheet, and stored after the agar gel and the silica gel were scraped and washed off.

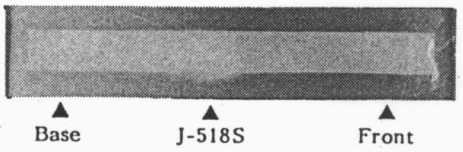

Figure 3-5. Zymoautography.
Chromatography was done with 10 μl of the partially purified J-518S substance (310 U) on a strip (2 × 20 cm) of a silicic-acid thin-layer plate (Chromagram) with ethyl acetate/methanol (3 : 2, v/v) as developing solvent. Inhibition of adenosine deaminase in the layer was judged by eye.

3.3. DISCUSSION

The change in colour of the assay plates from yellow or pale orange to purplish red during incubation is attributed to the pH indicator, which changes colour as ammonia is liberated during the deaminating reaction and as the pH increases. The change is fairly rapid and quite vivid, and its use in the assays described is of great practical value. It was difficult to decide exactly where the borders of the zones were, whether they were of activity or inhibition. Zone readings can, however, be done roughly, leading to the estimation of enzyme or inhibitor activities in the tested solutions. Figure 3-3b, which shows the case of an inhibitor, indicates that if one worker does all of the measurements, the inhibitory activity in the sample solution can be estimated by comparison of its diameter on a plot with that of a standard (*e.g.*, coformycin for adenosine deaminase inhibitor) with a series of known concentrations over a wide range of magnitudes. In particular, samples can be assayed, although only approximately, with one or a few paper disks for each sample, several for the standard if needed, and one agar plate. In contrast, although the usual spectrophotometric assay is precise, it involves several runs of assay reactions with the series of dilution of samples, and each run must be carried out separately. Although the number of diluted inhibitor samples may be reduced to a few for each assay by the use of log-probit plots [73], these runs are not easy to carry out, and are time-consuming. When the assay must be precise, an estimate obtained by the pH indicator assay would be a labour-saving preliminary to the spectrophotometric assay to follow.

The merit of this pH indicator method is that results are readily judged by eye. Its real usefulness is in the detection of the active fractions in column chromatography

of the deaminases and their inhibitors. As the size of the coloured zones are correlated linearly with the logarithms of the activity in the fractions, detection with a paper disk for each fraction covers a wide range of activity for several orders of magnitude. The active fractions can be detected in a belt of inhibition on the agar plates at a glance after a few hours of incubation. The width of the belt indicates the strength of the activity of the fraction. The method does not require constant attendance, but only preparation of the assay plates from stock lots of the assay mixture and the enzyme solution, application of paper disks wetted with sample solution to the surface of the agar, and occasional observation of the plates. Inhibitory zones can be read at any convenient time after they appear, because they are stable and do not change size after a certain time. In an enzyme activity assay, however, the zones must be read at the proper time, after which they become larger, finally interfering with each other and blurring as the reaction and the diffusion of the ammonia generated proceed.

The value of this method for detection of inhibitors is enhanced by its specificity for specific inhibitors only. Being a specific, visual method, it should be applicable for screening for these inhibitors. One Petri dish can contain several dozen samples (figure 3-4a). Specific inhibitors of adenosine deaminase will probably be used clinically as the co-agents of adenosine analogues (adenine pseudonucleosides), such as ara-A (9-D-arabinofuranosyladenine), which substances are inactivated by deamination with adenosine deaminase [71].

This new method will still be convenient and useful when employed to detect an enzyme in column chromatography. With this method, studies of cytosine deaminase are much easier.

3.4. EXPERIMENTAL

Unless otherwise noted, the experimental procedures are the same as in Chapter 2.

3.4.1. *Materials*

Chemicals. Adenosine was the product of the Yamasa Shoyu. Adenosine deaminase was the ADA [I] preparation of Boehringer Mannheim GmbH (Mannheim, F. R. G.) from calf intestinal mucosa, with a specific activity of about 200 U/mg. Coformycin was generously provided by Meiji Seika Kaisha (Tokyo). Agar was an analytical-grade product of the Kishida Chemical Co. (Osaka). DEAE-cellulose was type SH of the Brown Co. (Berlin, New Hampshire). Sephadex LH-20 was obtained from Pharmacia.

Other materials. Paper disks were 6 mm in diameter, made from thick filter paper (Toyo Roshi no. 526; 0.7 mm thick) with an office paper punch. Petri dishes (9 cmϕ) and rectangular culture plates (14 × 24 cm) had bottoms made of flat glass. Silica-gel thin-layer chromatography plates were commercial pre-coated plastic sheets (Chromagram 13179; Eastman Kodak Co., Rochester, N.Y.).

3.4.2. *Cytosine deaminase*

Cytosine deaminase from *E. coli* was a preparation partially purified by fractional precipitation with ammonium sulphate, as will be described in Chapter 4.

3.4.3. *J-518S substance*

J-518S seems to be a new specific inhibitor of adenosine deaminase, formed during the culture of an isolated streptomycete, J-518S, in the culture filtrate. A summary of purification procedures is shown in table 3-1.

Cultivation. The streptomycete was cultivated with a 10% inoculum at 30℃ for 2 days in a 20-L jar fermentor containing 10 L of a medium composed of 3% glucose, 0.2% yeast extract, 0.2% meat extract, 0.2% peptone, 0.05% KH_2PO_4, and 0.01% $MgCl_2$ in tap water (pH 8.3). The culture broth was collected by filtration with folded filter paper (8.5 L; pH 4.2). The filtrate was adjusted to pH 7.0 with NaOH.

Activated charcoal. To the filtrate was added 2% (w/v) activated charcoal, and the mixture was stirred for 10 min. The activity was eluted batchwise four times from the charcoal with 0.4 volume each time of 80% methanol in water, pH 8.0. The eluate was collected by decantation and concentrated to 1.56 L by evaporation under reduced pressure. The concentrate was adjusted to pH 7.0 with NaOH, and again treated with 2% (w/v) charcoal; elution was quite in the same way as above; then 2.5 L of the methanol extract was collected and concentrated to 275 ml, adjusted to pH 7.0; centrifuged, and microfiltered (0.3-μm pores) to remove the charcoal.

Ion-exchange column chromatography. The concentrate was put on a column (2ϕ × 32 cm) of Dowex 1 X8 (Cl^-) at the flow rate of 0.5 ml/min. The activity was not absorbed, but passed through the column. The column was washed with water. The solution obtained and the wash water was combined (420 ml), put on a column (2ϕ × 32 cm) of Dowex 50W X8 (NH_4^+), and eluted at the rate of 0.5 ml/min. The column was washed with water. Some activity was not absorbed. The solution that passed through and the wash water, which together contained 35% of the initial activity, was discarded. The activity that had absorbed was eluted with 0.2 M NH_4OH at the rate of 0.5 ml/min and 20-ml fractions were collected. The active fractions were combined (420 ml), evaporated under reduced pressure (to 44 ml), and adjusted to pH 7.0 with 10% ammonia.

Activated-charcoal column chromatography. The concentrate was chromatographed on a column (1ϕ × 19 cm) of activated charcoal at the flow rate of 0.2 ml/min. After the column was washed with water, it was eluted with methanol at the flow rate of 0.2 ml/min, and 12-ml fractions were collected. The active fractions (60 ml), which contained 28 mg of solid matter) were combined and concentrated to several millilitres by evaporation under reduced pressure.

Silicic-acid column chromatography. To the evaporator flask was added 0.50 g of silicic acid. The mixture was evaporated to dryness under reduced pressure and transferred to a column (2ϕ × 10 cm) of silicic acid with ethyl acetate. Chromatography was done with ethyl acetate/methanol (7 : 3, v/v), and 10-ml fractions were collected. The elution was monitored by the visual assay described here and reported in the text. The active fractions were collected (60 ml) and concentrated to 4 ml.

Gel-permeation chromatography. The molecular weight of the J-518S substance was estimated to be about 410 by gel-

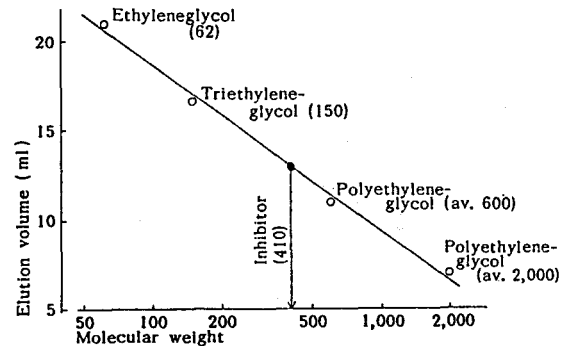

Figure 3-6. Graphic estimation of molecular weight of J-518S substance by Sephadex LH-20 gel permeation chromatography.

Table 3-1. Summary of purification of the J-518S substance.

Step	Volume (ml)	Weight (mg)	Activity ($\times 10^3$ U)	Yield (%)	Purification (fold)
Culture filtrate	8,500		13,000		
Activated charcoal 1	1,560	3,300	17,000	100	1
Activated charcoal 2	280	1,900	14,000	81	1.4
Dowex 1	420	590	8,700	51	2.8
Dowex 50W	430		2,900	17	
Activated charcoal 3	60	28	1,600	9.6	11
Silicic acid	67	8.8	1,000	5.9	22

permeation chromatography on a column (1ϕ × 21 cm) of Sephadex LH-20 after the method for proteins [1] with ethylene glycol (molecular weight of 62) and its polymers (molecular weights up to about 2,000 as a mean) as the standards (figure 3-6).

3.4.4. *Spectrophotometric assays*

Adenosine deaminase. The assay of adenosine deaminase activity employed a method modified from the direct spectrophotometric method based on the differential absorption of the substrate, adenosine, and of the product, inosine [40]. Activity was assayed by measurement of the decrease in absorbance at 265 nm resulting from the reaction. The standard assay system contained 0.04 μg (as protein) of adenosine deaminase, 0.15 μmol of adenosine, and 0.15 mmol of the buffer in 3.0 ml. The assay reaction was carried out at 30℃ in a recording spectrophotometer equipped with a thermoregulator. The photometer was set at 265 nm. After the solutions, each containing buffer and enzyme, were pipetted into a quartz cuvette with a 1-cm light path and warmed for several minutes in the spectrophotometer, the reaction was started by the addition of the warmed substrate solution, followed by rapid mixing; the reaction was run for 10 min. The changes in absorbance with time were recorded on a recorder, and its rate was read from a chart. One unit (U) of enzyme activity is defined as the amount needed to deaminate 1 μmol of adenosine per minute under these conditions. It corresponds to a change in absorbance at 265 nm of 2.8 absorbance units/min (ΔA_{265} of adenosine to inosine at pH 7.0 is 8,400 $M^{-1} \cdot cm^{-1}$).

Adenosine deaminase inhibitors. The inhibition of adenosine deaminase by a compound was measured in the standard assay system for adenosine deaminase. The compound was added to various concentrations in the assay mixture, and the reaction rates were read from the recorder. One unit (U) of inhibitory activity is defined as that inhibiting 50% of the enzyme activity under these conditions with 0.04 μg of adenosine deaminase/3 ml of assay mixture, and was calculated as for a microbioassay of antibiotics [73]. The concentration of the compound in the assay mixture at 50% inhibition that corresponds to 1 U per assay was read from a calibration curve made by the plotting of the percentage of inhibition of the reaction on a probit scale against the logarithm of the concentration.

3.4.5. *Assay plates*

Adenosine and cytosine deaminases. The components of the plates were mixed and heated to melt the agar. This mixture can be prepared in lots and stored in a refrigerator long term; when needed, a portion can be weighed off with a spoon, heated to melt the gel, and used. The agar solution was poured into Petri dishes or rectangular plates to a depth of about 2 mm, and allowed to cool to solidity. Paper disks were soaked in the standard solution, drops removed with filter paper, and the disks put onto the surface of agar plates. Incubation was at 30℃.

Adenosine deaminase inhibitors. When activity inhibiting adenosine deaminase was assayed, the solution of adenosine deaminase was added to the mixture after the mixture was cooled to about 50℃ and before gellation occurred.

3.4.6. *Photography*

Agar plates, made in glass-bottomed containers, were photographed on a light box made of a white translucent acrylic board and fluorescent lamps. For figure 3-2b, the photograph was taken under fluorescent lamps.

CHAPTER 4

Cytosine deaminase from *Escherichia coli* [c,d]

Different culture conditions for the production of cytosine deaminase from *Escherichia coli* were examined. A pH-stat culture with the culture pH kept at around 8.5 by the addition of citric acid gave efficient production in terms of cell mass and enzyme activity.

For purification to be efficient, use of affinity chromatography seemed to be suitable. Many classes of pyrimidine compounds were immobilized on the carrier, Sepharose 4B, *via* alkyl spacers by the construction of various spacers by cyanogen bromide activation of the carrier. Crude enzyme solutions were studied by adsorption and desorption chromatography with columns of the 68 kinds of gels thus obtained. Five gels were effective: (1) 2-Mercaptopyrimidine or (2) 2-thiobarbituric acid, when coupled with 1,6-diaminohexane and then with the activated carrier, was suitable as was (3) 2-amino-4,6-dihydroxypyrimidine or (4) 5-aminouracil, when linked by carbodiimide coupling to a carrier coupled with 6-aminohexanoic acid, and (5) orotic acid, when linked in the same way with a carrier coupled with 1,4-diaminobutane, was also effective.

The enzyme was purified 1200-fold from the cell extract, to homogeneity, by the usual procedures and by use of affinity chromatography with two of the above adsorbents; first with the immobilized 2-mercaptopyrimidine (1) and then with the immobilized 5-aminouracil (4). This enzyme had a molecular weight of 200,000 (estimated by gel permeation), and seemed to be an SH-enzyme. The purified enzyme was thermostable; achieving this property was the main objective of this study.

4.1. INTRODUCTION

I wanted to obtain enough cytosine deaminase with sufficient purity from *Escherichia coli* for the purpose of the possible clinical use described in the previous chapters. First, the conditions of the production of cytosine deaminase from *E. coli* were examined in order to improve production. *E. coli* was cultured in a tank under the optimum conditions established, and the enzyme was purified from the cell extracts. Preliminary experiments showed that the amount of the enzyme protein in the cell extract was very small and that purification on the scale of about a thousand times was necessary. For this purpose, I tried using affinity chromatography with pyrimidine compounds as the affinity ligands in addition to the usual steps of fractional precipitation and chromatography. Sepharose 4B was the carrier and many methods already in use were adopted without modification. Two adsorbents were selected and used in purification.

This chapter also deals with the characteristics of the purified enzyme.

4.2. RESULTS

4.2.1. *Production of cytosine deaminase in culture of* E. coli

To find the optimum culture conditions for the production of cytosine deaminase in the culture of *E. coli*, the following experiments were done.

4.2.1.1. *Effect of carbon source*

Several compounds were used instead of glycerol in the growth medium at the same concentration in terms of carbon. Culture was in 500-ml shaking flasks. After 24 hr, the cells were harvested and cell extracts were obtained. The enzyme activity in the extracts is shown in table 4-1. Citric acid gave excellent results, so it was used as the main carbon source in later experiments.

Table 4-1. Effects of carbon sources on formation of cytosine deaminase in culture of E. coli.

Carbon source	(%)	Culture		Enzyme activity (U/ml culture)
		OD_{610}	pH	
Glycerol	1.00	20.5	7.4	0.004
Glucose	0.98	21.7	7.5	0.006
Sucrose	0.93	20.0	7.5	0.004
Starch	0.88	18.8	7.8	0.005
Acetic acid (Na salt)	1.34	Sparse growth		
Citric acid (Na_3 salt $2H_2O$)	1.60	18.4	8.1	0.012
Succinic acid (Na_2 salt $6H_2O$)	2.20	16.8	8.0	0.004
Fumaric acid (NaH salt)	1.12	19.3	8.2	0.003
Tartric acid (Na_2 salt $2H_2O$)	1.88	20.9	7.9	0.004
Oxalic acid (Na_2 salt)	2.18	8.5	8.1	0.004
None		18.0	7.9	0.002

4.2.1.2. *Effect of nitrogen source*

Different nitrogen sources were used in a growth medium containing 1.04% citric acid in place of 1% glycerol and 0.2% yeast extract instead of 0.6%. Several compounds were substituted for peptone (3%), and culture was for 24 hr. The specific activity was highest when 1% aspartic acid was used, but peptone at the concentration of 3% promoted growth better and resulted in the production of the most enzyme activity (table 4-2). This medium, with 1.04% citric acid, 0.2% yeast extract, and 3% peptone, is referred to as the optimum medium.

4.2.1.3. *Effect of culture pH*

The pH of the culture increased during cultivation (table 4-2); it seemed that pH might affect growth and enzyme

Table 4-2. Effects of nitrogen source on formation of cytosine deaminase in culture of E. coli.

Nitrogen source	(%)	Culture		Enzyme activity	
		OD_{610}	pH	(U/ml culture)	(U/mg protein)
Peptone	3	23.3	8.1	0.018	0.10
	1	14.3	8.7	0.005	0.04
Meat extract	3	12.1	8.6	0.00	0.11
	1	8.8	8.8	0.008	0.29
Yeast extract	1[a]	11.6	8.7	0.009	0.17
Aspartic acid	1	11.6	8.7	0.009	0.38
Urea	1	8.9	8.9	0	—
Ammonium citrate	1[b]	10.1	8.7	0.003	0.20
Ammonium sulphate	1	6.1	8.7	0	—
Ammonium chloride	1	6.9	8.6	0	—

[a] The final concentration of the yeast extract was 1.2%.
[b] Citric acid was present at 1.6% as the free acid.

production. In preliminary experiments, enzyme formation increased when the pH of the culture was controlled with citric acid. Therefore, this acid was added to the culture as an additional growth substrate so that the pH of the culture would not exceed a certain value with use of a pH-stat. A pH

Table 4-3. Production of cytosine deaminase in E. coli *cultured with use of pH-stat.*

Culture no.	Culture conditions			Maximum growth			Maximum enzyme activity					
	Citric acid in culture medium at 0 hr [A] (mol/L; g/dl)	Control of pH		Time	OD_{610}	Enzyme activity	Time	OD_{610} [B]	Enzyme activity [C]	Specific activity [C/B]	Citric acid supplied[a]	
		pH fixed	Acid used	(hr)		(U/ml)	(hr)		(U/ml)	(U/ml per OD_{610})	from pH controller [D] (mol/L; g/dl)	Sum [A+D] (mol/L; g/dl)
1	0.15; 1.04	7.0	Citric	27[b]	32	0.007	15	15	0.008	0.0006	0.37; 2.6	0.52 3.6
2	0.15; 1.04	7.5	Citric	51	30	0.056	48	29	0.063	0.0022	0.48; 3.3	0.62; 4.4
3	0.15; 1.04	8.0	Citric	24[b]	32	0.039	24	32	0.039	0.0012	0.55; 3.8	0.70; 4.4
4	0.15; 1.04	8.5	Citric	36	25	0.105	36	25	0.105	0.0042	0.20; 1.4	0.35; 2.4
5	0.15; 1.04	8.5	Citric	51	44	0.030	30	37	0.053	0.0014	0.25; 1.7	0.40; 2.8
6	0.15; 1.04	8.5	Citric	39[b]	66	0.101	36	60	0.105	0.0017	0.56; 3.9	0.71; 5.0
7	0.15; 1.04	8.5	Citric	24	25	0.026	21	25	0.089	0.0036	0.61; 4.3	0.76; 5.4
8	0.15; 1.04	9.0	Citric	18	30	0.022	15	30	0.022	0.0007	0.11; 0.8	0.26; 1.8
9	0.15; 1.04	9.0	Citric	36	43	0.038	27	36	0.040	0.0011	—[c]	—[c]
10	0.15; 1.04	8.5	HCl	15	30	0.024	15	30	0.024	0.0008[d]	0.06; (HCl)	0.21; 0.15[d]
11	0; 0	8.5	HCl	15	43	0.003	9	31	0.006	0.0002	0.05; —	0.05; 0[d]
12	0.15; 1.04	(No control)		21[e]	24	0.014	18[f]	16	0.020	0.0013	0; 0	0.15; 1.0

[a] Up to the time of maximum enzyme activity. [b] Growth was still continuing. [c] Data not obtained. [d] Amount of citric acid only. [e,f] pHs were [e] 8.9 and [f] 8.6 at the time.

controller was attached to the mini-jar fermentor for this purpose, and *E. coli* was cultivated at 30℃ in the fermentor containing the optimum medium. After the pH reached the desired value, it was automatically controlled with 50% citric acid solution. Every 3 hr, samples were withdrawn and the OD_{610} and pH of the samples were measured. When necessary, enzyme activity of the cells in the samples was measured after the cells were collected and sonicated. Table 4-3 shows the results of independent single cultures at various pH (culture nos. 1~9, with culture nos. 10~12 as controls). The specific activity of the enzyme is expressed in units/millilitre per OD_{610} of the culture. The enzyme activity was highest at the late stationary phase. The data in table 4-3 are not very precise, but control of the culture pH with citric acid to around 8.5 was effective. The profile of culture no. 6 is shown in figure 4-1.

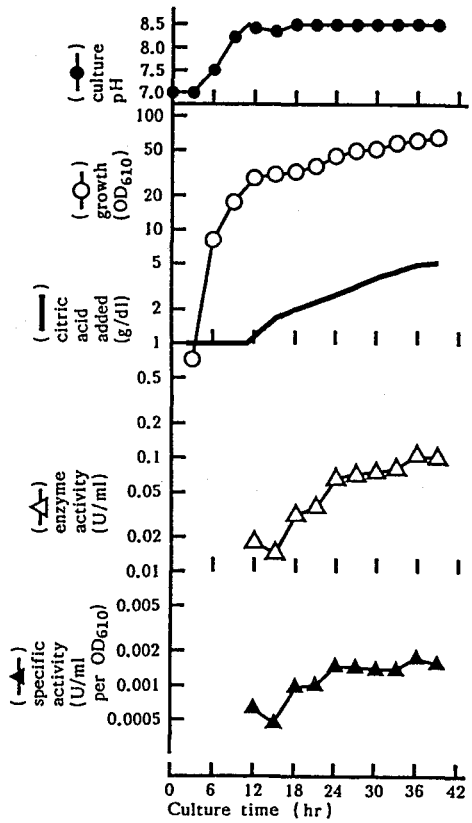

Figure 4-1. Example of a pH-stat culture at pH 8.5 with citric acid.
The profile of culture no. 6 in table 4-3 is shown.

4.2.2. *Affinity chromatography of* E. coli *cytosine deaminase*

Affinity chromatography was examined if it could be used for purification of cytosine deaminase from *E. coli*.

Sepharose. The carrier selected for immobilization of the affinity ligands was Sepharose 4B, a beaded agarose gel, called Sepharose here.

CNBr-activated Sepharose. Sepharose was activated with CNBr (cyanogen bromide) by an improved and simplified

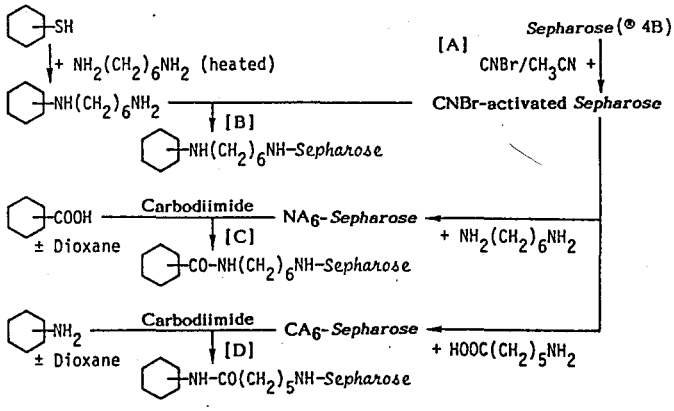

Figure 4-2. Outline of CNBr-activation of [A] *Sepharose and immobilization of* [B] *mercapto-,* [C] *carboxy-, and* [D] *amino-pyrimidines on the product thus activated.*
Hexagons represent pyrimidine rings.

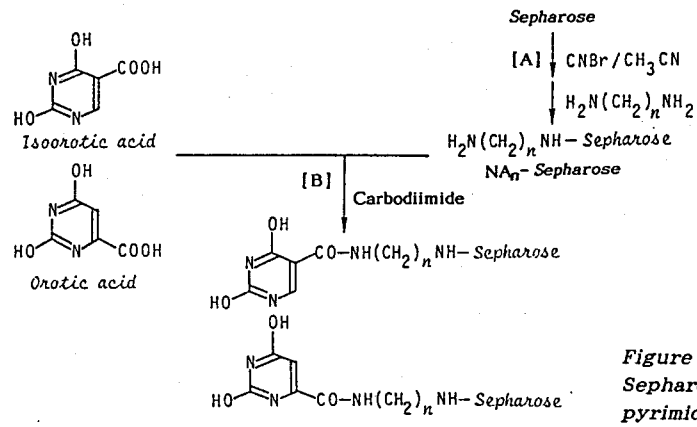

Figure 4-3. Outline of preparation of NA$_n$-Sepharose [A] *and immobilization of carboxypyrimidines* via *long spacers* [B].

method that uses acetonitrile, and then immediately used for the coupling reaction [51] (figure 4-2,A). This procedure is referred to below as "reaction with CNBr-activated Sepharose" or the like.

4.2.2.1. *NA-Sepharoses*

The first group of adsorbents were ω-aminoalkylimino-Sepharoses with spacer arms *n* carbon atoms long (NA$_n$-Sepharoses; figure 4-3,A). Many kinds of such Sepharoses in which *n* was 2, 3, 4, 5, 6, 7, 8, 9, 10, and 12 were synthesized (gel nos. 1~10), and a cell extract was chromatographed on columns of these gels. Elution of the enzyme from the columns was done with a stepwise gradient of potassium chloride in the elution buffer (table 4-4). Some of the gels gave good yields, although with the extent of purification was slight.

Table 4-4. Column chromatography of the cell extract of E. coli *on NA-Sepharoses.*

NA$_n$-Sepharoses (10 ml each) were packed in columns (1 cmϕ). The cell extract (10 ml, containing 2.0 U of activity and 42 mg of protein) was dialysed against 10 mM buffer, and put on each column; the column was washed with the same buffer. Then chromatography was done with a stepwise concentration gradient of KCl in buffer (50-mM increase per 5-ml fraction).

—, Not measured (and not calculated).

Gel no.	*n*	Active fraction			Purification (fold)	Recovery (%)
		KCl concentration (mM)	Activity (U)	Protein (mg)		
1	2	No adsorption[a]				
2	3	No adsorption[a]				
3	4	No adsorption[a]				
4	5	150, 200	2.0	10	4.0	96
5	6	200, 250	0.88	3.6	5.0	43
6	7	200	1.5	8.1	3.8	74
7	8	150, 200	1.2	—	—	—
8	9	No adsorption[a]				
9	10	No adsorption[a]				
10	12	100, 150	1.5	5.2	6.1	76

[a] Enzyme activity passed through the column without adsorption.

4.2.2.2. *Immobilized mercaptopyrimidines*

The second group of adsorbents were mercaptopyrimidines immobilized on Sepharose with a C$_6$ spacer (figure 4-2,B). This procedure followed the procedure reported for immobilization of ATP [17]. The ligands were coupled by replacement reactions with 1,6-diaminohexane as the spacer, and further coupled with CNBr-activated Sepharose *via* the resulting amino termini (gel nos. 11~15). The crude enzyme solution was chromatographed on columns of the completed gels (table 4-5). 2-Mercaptopyrimidine (gel no. 11) and 2-thiobarbituric acid (gel no. 12) gave gels that could be used for purification and also allowed 100% recovery of activity.

Table 4-5. Column chromatogaphy of the cell extract on mercaptopyrimidines immobilized on Sepharose via C_6 spacers.

Mercaptopyrimidines (40 μmol each) were coupled with 1,6-diaminohexane (0.40 mmol) under the conditions given and allowed to react with CNBr-activated Sepharose (10 ml) as described in the experimental section. The completed gels were packed in columns (1 cmφ). The cell extract containing 0.75 U of activity and 140 mg of protein in 10 ml was dialysed against 10 mM buffer and put on the columns. The columns were washed with the same buffer. Then chromatotography was done with buffer with a stepwise gradient of KCl (100-mM increase per 10-ml fraction).

1

2

3

4

Gel no.	Ligand	Coupling conditions		Active fraction			Purification (fold)	Recovery (%)
		Temperature (℃)	Time (hr)	KCl[a] (mM)	Activity (U)	Protein (mg)		
11	2-Mercaptopyrimidine	80	6	200	0.75	15	9.6	100
12	2-Thiobarbituric acid[b]	90	12	200	0.75	17	8.5	100
13	5-Mercaptouracil[c]	80	12	200	0.51	17	3.8	68
14	2-Thiouracil[d]	120	12	300	0.33	17	3.8	45
15	2-Thiocytosine[e]	140	16	300	0.30	15	3.8	40

[a] KCl concentration in the eluate. [b] 2-SH-4,6-$(OH)_2$-, [c] 2,4-$(OH)_2$-5-SH-; [d] 2-SH-4-OH-; and [e] 2-SH-4-NH_2-pyrimidine.

4.2.2.3. *Immobilized carboxy- and aminopyrimidines*

The third and fourth groups of adsorbents were carboxy- (gel nos. 16~20; figure 4-2,C) and aminopyrimidines (gel nos. 21~30; figure 4-2,D) immobilized on NA_6- and CA_6-Sepharose (6-aminohexylimino- and 5-carboxypentylimino-Sepharose), respectively, by carbodiimide coupling. Chromatography of the crude enzyme solution was done with columns of these adsorbents. The immobilized carboxypyrimidines tested (gel nos. 16~20) gave poor results (figure 4-4); most of the immobilized aminopyrimidines tested (gel nos. 22~24, 26~30)

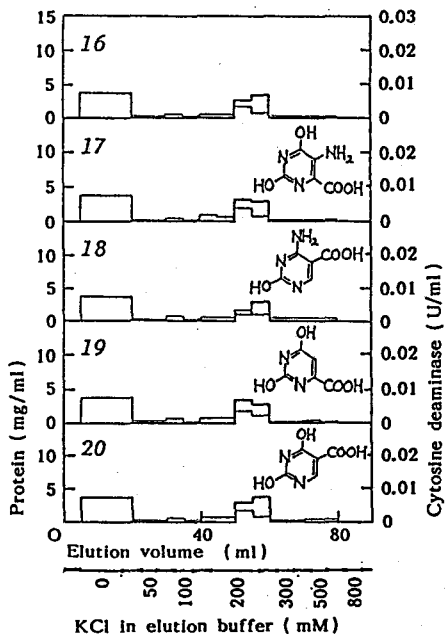

Figure 4-4. Column chromatogaphy of the cell extract on carboxypyrimidines immobilized on NA_6-Sepharoses.

A pyrimidine compound (0.20 mmol) was reacted with NA_6-Sepharose (10 ml) by carbodiimide coupling in the presence of 50% (v/v) dioxane. The completed gels were packed in columns (1 cmφ) and used in chromatography of the cell extract (5.0 ml, containing 1.7 U of activity and 68 mg of protein) as above. The columns were washed with elution buffer (10 ml) containing 50 mM KCl. Then elution was done with 100 mM KCl.

Gel no.	Ligand	Active fraction		Purification (fold)	Recovery (%)
		Activity (U)	Protein (mg)		
16	None[a]	0.62	13	2.1	39
17	5-Carboxycytosine	0.45	13	1.5	28
18	Isoorotic acid	0.70	14	2.2	44
19	Orotic acid	0.66	15	1.8	41
20	5-Aminoorotic acid	0.66	13	2.2	41

[a] An preparation of NA_6-Sepharose (prepared separately from gel no. 5 in table 4-4) was used.

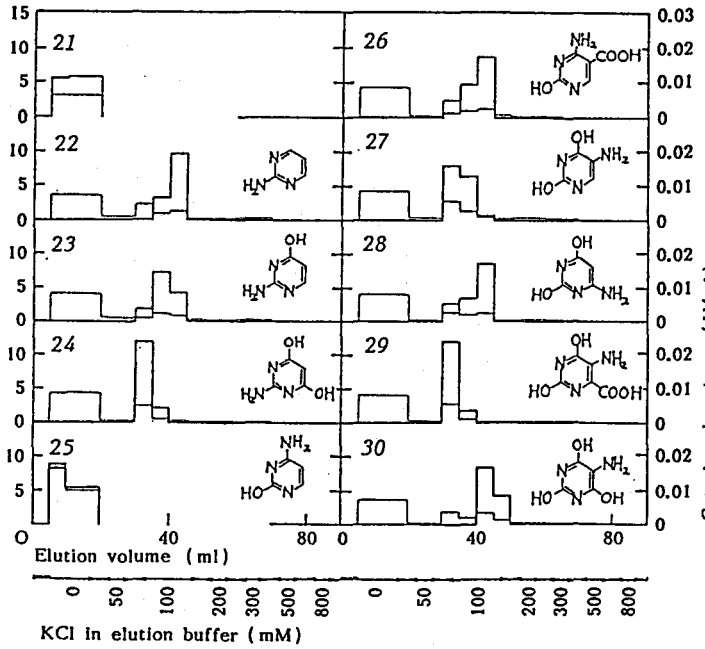

Figure 4-5. Column chromatography of the cell extract on aminopyrimidines immobilized on CA6-Sepharoses.

A pyrimidine compound (0.20 mmol) was reacted with CA6-Sepharose (10 ml) in the same way as in the legend to figure 4-4. The completed gels were used in the chromatography of the cell extract used there. In gel nos. 21 and 25, lines are missing where the stepwise gradient elution was not done.

Gel no.	Ligand	Active fraction		Purification (fold)	Recovery (%)
		Activity (U)	Protein (mg)		
21	None[a]	No adsorption			
22	2-Aminopyrimidine	0.99	6.3	6.7	62
23	Isocytosine	1.1	11	4.7	72
24	2-Amino-4,6-dihydroxypyrimidine	1.4	16	4.0	91
25	Cytosine	No adsorption			
26	5-Carboxycytosine	1.4	11	5.5	85
27	5-Aminouracil	1.5	17	3.7	92
28	6-Aminouracil	1.2	10	4.8	74
29	5-Aminoorotic acid	2.3	13	3.8	76
30	Uramil	1.3	11	4.8	81

[a] CA6-Sepharose was used as in the chromatography (not in preparation of affinity gel without ligand).

were more effective than the above carboxypyrimidines for purification and gave better recovery (figure 4-5). Immobilized 2-amino-4,6-dihydroxypyrimidine (gel no. 22) and 5-aminouracil (gel no. 27) gave the best recovery of enzyme activity, with some purification.

4.2.2.4. *Carboxypyrimidines immobilized* via *spacers of various lengths*

The fifth group of adsorbents were carboxypyrimidines immobilized on NA$_n$-Sepharoses with various *n* numbers (figure 4-3,B). Isoorotic acid was coupled with NA$_n$-Sepharoses by carbodiimide coupling (gel nos. 31～40), and was orotic acid (gel nos. 41～50) in the same way as the third group of adsorbents (gel nos. 18 and 19). Chromatography of the enzyme on columns of these gels was done (table 4-6). The four columns of gels prepared from both ligands with C_4 and C_{12} spacers (gel nos. 33, 40, 43, and 50) resulted in good recovery of activity with considerable purification.

4.2.2.5. *Aminopyrimidines immobilized* via *long spacers*

The sixth group of adsorbents were aminopyrimidines immobilized on Sepharose with carboxy-terminated long spacers. These long spacers were constructed on Sepharose as follows (figure 4-6,A). α,ω-Dicarboxyalkanes *m* carbon

Table 4-6. Column chromatography of the cell extract of E. coli *on carboxypyrimidines immobilized on NA-Sepharoses.*

Isoorotic or orotic acid (0.20 mmol) was allowed to react with one of the NA_n-Sepharoses listed (10 ml) by carbodiimide coupling. The completed gels were packed in columns (1 cmϕ), and used in chromatography of the same cell extract as described in the headnote of table 4-4.

	Ligand											
	Isoorotic acid						Orotic acid					
n	Gel no.	Active fraction(s)			Purification (fold)	Recovery (%)	Gel no.	Active fraction(s)			Purification (fold)	Recovery (%)
		KCl concentration (mM)	Activity (U)	Protein (mg)				KCl concentration (mM)	Activity (U)	Protein (mg)		
2	*31*	No adsorption					*41*	No adsorption				
3	*32*	No adsorption					*42*	No adsorption				
4	*33*	100	1.3	4.0	7.2	68	*43*	100, 150	2.1	8.2	5.8	113
5	*34*	200, 250	0.56	—	—	29	*44*	200, 250	1.1	—	—	58
6	*35*	150, 200	0.47	—	—	25	*45*	200, 250	0.73	—	—	39
7	*36*	200, 250	0.85	—	—	45	*46*	200, 250	0.77	—	--	41
8	*37*	200, 250	0.36	—	—	19	*47*	200, 250	0.71	—	—	38
9	*38*	No elution[a]					*48*	No elution[a]				
10	*39*	No elution[a]					*49*	No elution[a]				
12	*40*	100, 150	1.4	6.1	5.3	77	*50*	100, 150	0.99	3.5	6.4	53

[a] No enzyme activity was found in the eluate when the cell extract was put on the column, and no activity was eluted from the column under the conditions used.

atoms long (including carboxy carbons) were linked to NA_n-Sepharoses by cardodiimide coupling. The resulting conjugates were ω-(ω'-carboxyalkanoylimino)alkylimino-Sepharoses, called CA_m-NA_n-Sepharoses here. Carbodiimide reactions again coupled the amino ligands of 2-aminopyrimidine (gel nos. 51～59) and 5-aminouracil (nos. 60～68) to the carboxy termini, completing preparation of the adsorbents (figure 4-6,B). Chromatography of the enzyme on columns of these gels was done (table 4-7). The recovery of enzyme activity from the columns was poor, as was the purification effect calculated for several columns. This class of spacers was not useful.

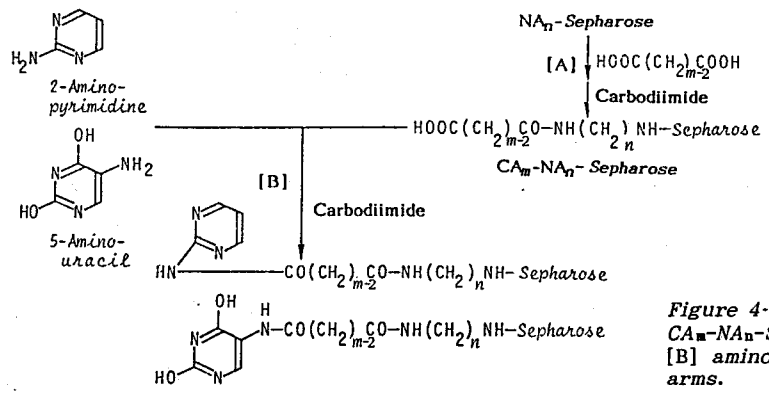

Figure 4-6. Outline of preparation of [A] *CA_m-NA_n-Sepharose and immobilization of* [B] *aminopyrimidines via the long spacer arms.*

4.2.2.6. *Gradient elution*

An example is given of chromatography by elution with a linear gradient of salt (figure 4-7). The 10 ml of crude enzyme solution contained 0.45 U of activity (with specific activity of 0.0018 U/ml per OD_{280}), and was put on a column of immobilized 2-mercaptopyrimidine described above. Protein that did not adsorb was washed out with the buffer and the enzyme was eluted with a linear gradient of potassium chloride (0 to 1,000 mM, 300 ml) in the buffer. The active fraction of eluates, with a volume of 10 ml, contained 0.49 U of activity (specific activity of 0.034 U/ml per OD_{280}), showing a 19-fold increase in specific activity over the crude enzyme and an overall yield of 110%. Other examples of chramatography with elution with linear gradients of salt will be described later as part of a purification procedure (figures 4-8,9).

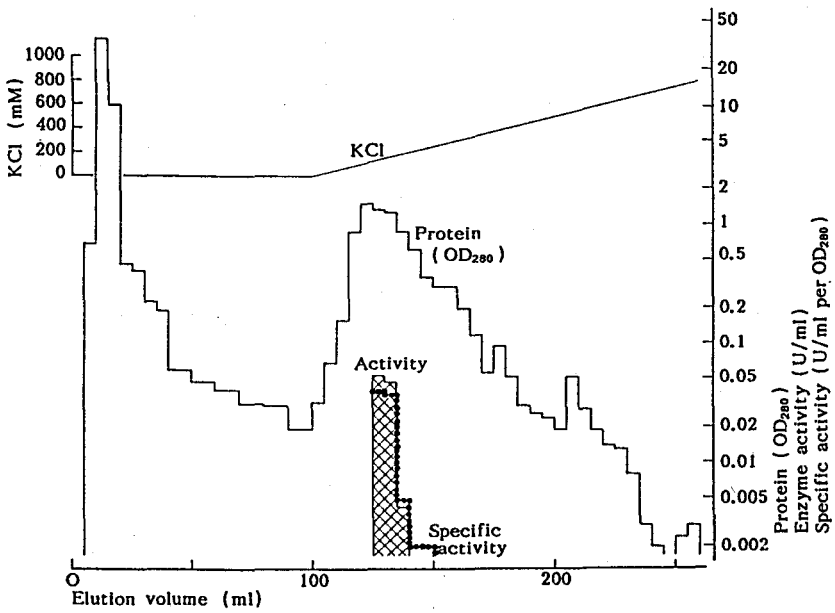

Figure 4-7. Column chromatography of the cell extract on immobilized 2-mercaptopyrimidine with a linear gradient of salt.

Table 4-7. Column chromatography of the cell extract of E. coli *on aminopyrimidines immobilized on CA_m-NA_n-Sepharoses.*

CA_m-NA_n-Sepharoses were prepared from CNBr-activated Sepharose by being coupled with an α,ω-diaminoalkane (n=2, 6, or 8; 2 mmol/10 ml of gel) and then reacted with an a,ω-dicarboxyalkane (m=6, 8, or 10; 2 mmol/10 ml of gel) by carbodiimide coupling without dioxane. 2-Aminopyrimidine or 5-aminouracil (0.2 mmol) was coupled with these CA_m-NA_n-Sepharoses (10 ml) in the same way. The completed gels were packed in columns (1 cmϕ). The same cell extract as in table 4-4 (10 ml each) was put into the columns. After the columns were washed thoroughly with the 10 mM buffer, elution was done with the same buffer containing 200 mM KCl (20 ml for each column).

		Ligand					
		2-Amino-pyrimidine			5-Amino-uracil		
m	*n*	Gel no.	Activity (U)	Recovery (%)	Gel no.	Activity (U)	Recovery (%)
6	2	*51*	n.a.[a]		*60*	n.a.[a]	
8	2	*52*	n.a.[a]		*61*	n.a.[a]	
10	2	*53*	n.a.[a]		*62*	n.a.[a]	
6	6	*54*	0.87	47	*63*	0.51	27
8	6	*55*	0.81	47	*64*	0.61	32
10	6	*56*	0.18	10	*65*	0.40	22
6	8	*57*	0.14	8	*66*	0.45	24
8	8	*58*	0.16	9	*67*	0.45	24
10	8	*59*	n.e.[b]		*68*	n.e.[b]	

[a] n.a., No adsorption; [b] n.e., no elution.

4.2.3. *Purification of* E. coli *cytosine deaminase*

The enzyme was purified from the cell extract prepared

Table 4-8. Purification of cytosine deaminase from E. coli.

Step	Protein (mg)	Activity (U)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
Cell extract	170,000	1,400	0.0081	1	100
Heat treatment	88,000	1,300	0.015	1.9	96
Ammonium sulphate	10,000	990	0.097	12	71
DEAE-cellulose SH	1,000	710	0.69	85	51
Immobilized 2-mercaptopyrimidine	290	490	1.7	210	35
Immobilized 5-aminouracil	73	360	5.0	610	26
Sephacryl S-300S	16	160	9.8	1,200	11

from 150 L of the culture as follows and as summarized in table 4-8.

4.2.3.1. *Tank culture and cell extraction*

The organism was cultivated in two batches in a triple jar fermentor containing 25 L of optimum medium in each vessel. After the pH of the cultures reached 8.5, it was kept at that value by a pH-stat with addition of 50% citric acid. Culture was stopped at 24 hr, when growth was in the late stationary phase; the OD_{610} was about 20. Cells were harvested by continuous centrifugation at 13,000 × *g*, washed twice with saline by centrifugation, and suspended in the buffer (total of 10 L in each batch). The cells in the suspension were disintegrated in a glass-bead mill to obtain cell extract.

4.2.3.2. *Heat treatment*

The cell extracts were separately heated by being pumped through a long glass spiral tube immersed in a circulating water bath maintained at 60℃ into a reservoir that was also in the bath. They were kept at this temperature for 30 min, and then cooled in an ice bath in a similar way; the denatured protein was removed by centrifugation for 20 min at 13,000 × *g*.

4.2.3.3. *Ammonium sulphate fractionation*

The enzyme was then precipitated by ammonium sulphate in the fraction between 30 and 55% saturation. The two

precipitates were collected by centrifugation and dissolved in the buffer.

4.2.3.4. *DEAE-cellulose column chromatography*

The enzyme solutions from the previous step were dialysed overnight against the buffer and centrifuged to remove the precipitates formed. The clarified solutions were combined, divided into two parts, and purified separately by chromatography on a column of DEAE-cellulose (4ϕ × 35 cm) equilibrated with the buffer. Protein that did not adsorb was thoroughly washed with the buffer, and elution was done with a linear gradient of potassium chloride (from 0 to 500 mM; total volume of 900 ml in one part) in the buffer. The active fractions were collected and dialysed overnight against the buffer.

4.2.3.5. *Affinity chromatography*

The two dialysed active fractions from the previous step were combined and put on a column (2ϕ × 30cm) of 2-mercaptopyrimidine immobilized on Sepharose equilibrated with the buffer. Chromatography was done in a similar way except for the total volume of eluant, 800 ml (figure 4-8). The active eluate fractions were collected and dialysed against the buffer. There was a 2.4-fold increase in specific activity

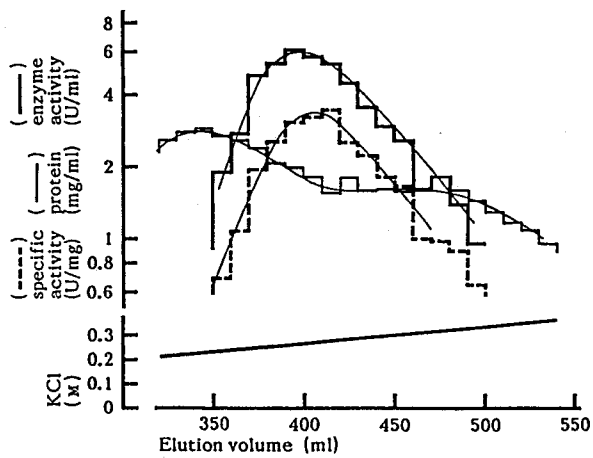

Figure 4-8. Affinity chromatography of a partially purified cytosine deaminase from E. coli *on another column of the immobilized 2-mercaptopyrimidine for purification.*
Details are given in the text.

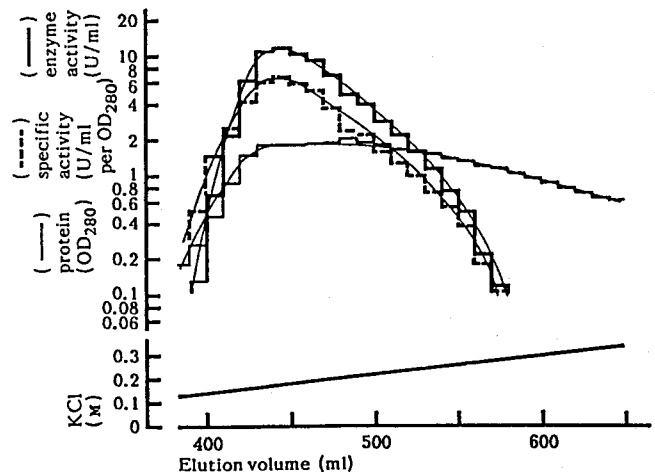

Figure 4-9. Second affinity chromatography of the partially purified cytosine deaminase from E. coli *with use of 5-aminouracil immobilized on CAs-Sepharose.*

compared to the enzyme put on the column, and an overall yield of 69%.

The dialysed solution was then chromatographed in a similar way on a column (1.5ϕ × 28 cm) of 5-aminouracil immobilized on Sepharose (figure 4-9). The active fractions were collected and concentrated by ultrafiltration; there was a 3.0-fold purification, with a 75% yield.

4.2.3.6. *Sephacryl S-300S column chromatography*

The final concentrate was dialysed against the 50 mM buffer containing 1 M potassium chloride, and studied by gel permeation on a column (1.5ϕ × 85 cm) of Sephacryl S-300S as described in the experimental section. The active fractions were combined, making 6.0 ml.

4.2.3.7. *Homogeneity*

Second ammonium sulphate fractionation. One-fifth (1.2 ml) of the above fraction was further purified by precipitation twice with ammonium sulphate in a conical centrifuge tube. Precipitation occurred at about 35% saturation. The final precipitate was collected by centrifugation and dissolved in about 0.5 ml of the buffer; only 11% of the activity before the precipitation was recovered. The specific activity of the enzyme was 11 U/ml per OD_{280} before and 10 after the repeated precipitation (not expressed in U/mg of protein).

Ultracentrifugation. This enzyme solution was analysed in an ultracentrifuge in 0.1 M potassium chloride in the 10 mM buffer as described elsewhere for the *Serratia* enzyme [64]. The enzyme sedimented as a single symmetric peak, which was blurred and low even at the lens angle of 55°, because of the low concentration of the enzyme (figure 4-10). The $s_{20,w}$ was calculated to be 14S.

Disc gel electrophoresis. The enzyme solutions before and after the second ammonium sulphate precipitation were tested by discontinuous polyacrylamide gel electrophoresis. The solution before precipitation had two bands, one sharp and intense and the other faint at the side of low molecular weights; the solution afterwards had a single band at the same place as the main band of the solution before precipitation (figure 4-11a). The results of this analysis suggested that the final enzyme preparation was a homogeneous protein.

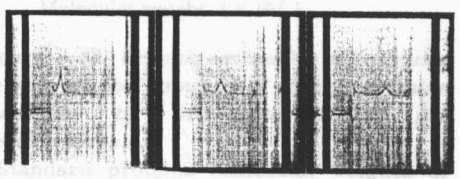

Figure 4-10. Ultracentrifugation of the purified E. coli *cytosine deaminase.*
Details are in the text. Rotation was at 52,000 rpm. Photographs were taken 5, 15, and 30 min (left to right) after the maximum speed was reached.

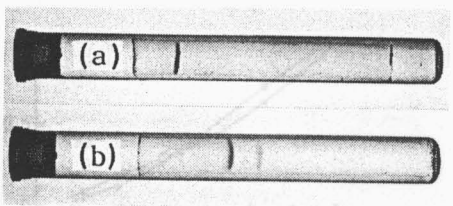

Figure 4-11. Polyacrylamide gel electrophoresis of E. coli *cytosine deaminase.*
Details are in the text. Electrophoresis was done in the (a) absence and (b) presence of SDS. Migration was from left (+) to right (-).

4.2.4. *Characteristics of* E. coli *cytosine deaminase*

The remaining Sephacryl fraction was used in the following studies of the characterization of the enzyme, except for the estimation of molecular weight.

4.2.4.1. *Molecular weight*

The column of Sephacryl S-300S used in the purification described above was calibrated, and the molecular weight of the enzyme was estimated from its point of elution [1] to be about 200,000 (figure 4-12). SDS-polyacrylamide gel electrophoresis gave two clear bands (figure 4-11b). These bands were compared graphically on a calibration curve with those of standard proteins that were simultaneously electrophoresed on several gels rods, and found to correspond to 35 and 46 kDa (figure 4-13). These results suggested that the molecule of the enzyme protein was composed of 35- and 46-kDa subunits.

4.2.4.2. *Isoelectric point*

Isofocusing electrophoresis of the enzyme gave the isoelectric point of about pH 5.8 (figure 4-14).

4.2.4.3. *Substrate specificity*

Some pyrimidine compounds (at 3 mM) were tested as substrates of the enzyme (0.0014 U/ml). Cytosine, 5FC, and 5-methylcytosine (5MC) were deaminated. The ratio of the activities was 1:0.7:0.1. Other compounds, including cytidine, cytidine 5′-monophosphate, adenine, adenosine, and adenosine 5′-monophosphate, were not substrates.

4.2.4.4. *Effects of heat*

The enzyme was incubated with cytosine at various temperatures (figure 4-15a). Enzyme activity was enhanced at over 37℃ and the optimum temperature for the reaction

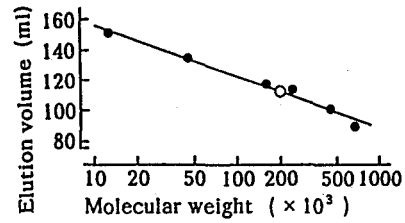

Figure 4-12. Graphic estimation of molecular weight of E. coli *cytosine deaminase by Sephacryl S-300S gel permeation chromatography.*

Details are given in the experimental section.

Standard proteins (molecular weight): horse heart cytochrome *c* (12,500); ovalbumin (45,000); rabbit muscle aldolase (158,000); bovine liver catalase (240,000); horse spleen ferritin (450,000); bovine thyroglobulin (669,000). Blue Dextran 2000 (2,000,000).

○, Cytosine deaminase; ●, standard proteins.

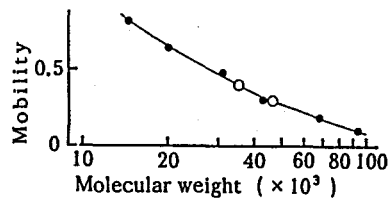

Figure 4-13. Graphic estimation of molecular weights of the subunits of E. coli *cytosine deaminase by SDS-polyacrylamide gel electrophoresis.*

Standard proteins (molecular weights of the proteins or subunits): hen egg-white lysozyme (14,400); soybean trypsin inhibitor (20,100); bovine erythrocyte carbonic anhydrase (30,000); ovalbumin (43,000); bovine serum albumin (67,000); human erythrocyte phosphorylase B (94,000).

○, Cytosine deaminase subunits; ●, standard proteins.

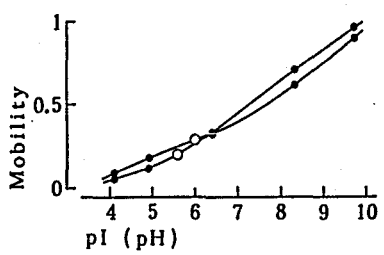

Figure 4-14. Graphic estimation of pI of E. coli *cytosine deaminase by isofocusing electrophoresis.*

The data from two runs are plotted. Details are in the experimental section.

○, Cytosine deaminase; ●, pI markers.

was around 50℃. To evaluate its thermostability, the enzyme was reacted with cytosine after incubation in the buffer for 6 hr at various temperatures. The activity was enhanced when incubation was over 37℃, and activity was stable up to about 55℃ (figure 4-15b).

4.2.4.5. *Effects of pH*

The activity of the enzyme at different pH was investigated (figure 4-16a). The optimum for the reaction was around pH 9. The effect of pH on the stability of the enzyme was examined by measurement of the activity after incubation at different pH at 37℃ for 6 hr (figure 4-16b). Preliminary incubation activated the enzyme. In this case, the activity was most stable at from pH 9 to 10.

4.2.4.6. *Effectors*

Metal ions. The effect of metal ions on the enzyme activity was examined (table 4-9a). Like most SH-enzymes [11], this enzyme was inhibited by very low concentrations of Ag^{+}, Cu^{2+}, Hg^{+}, or Hg^{2+}.

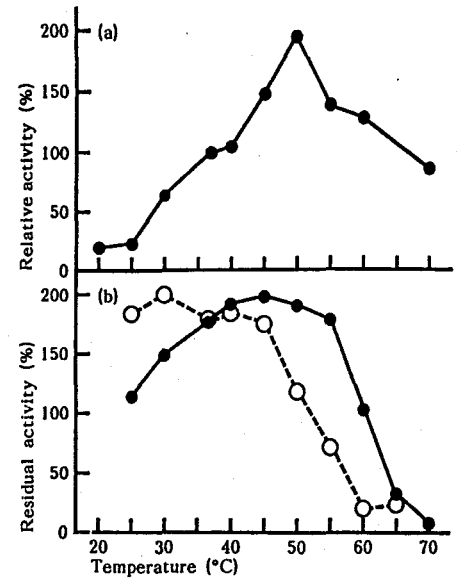

Figure 4-15. Effects of heat on (a) *the activity of the* E. coli *enzyme and* (b) *its stability.*

(a) *Optimum temperature.* The assay mixture (with 0.011 U of activity/ml) was incubated under the standard conditions except for temperature. The activity is expressed relative to that at 37℃.

(b) *Thermostability.* The enzyme (with 0.0074 U of activity) was incubated at the indicated temperature for 6 hr in 0.5 ml of phosphate or borate buffer (10 μmol). The reaction mixture was immediately cooled in an ice bath, and 0.2 mmol of phosphate buffer (pH 8.0) was added. The mixture was incubated at 37℃ for several minutes, and the assay reaction was started by the addition of cytosine to a total volume of 1.0 ml. The activity that remained is expressed relative to that of the untreated enzyme.

●, Phosphate (pH 8.0); ○, borate (pH 9.5).

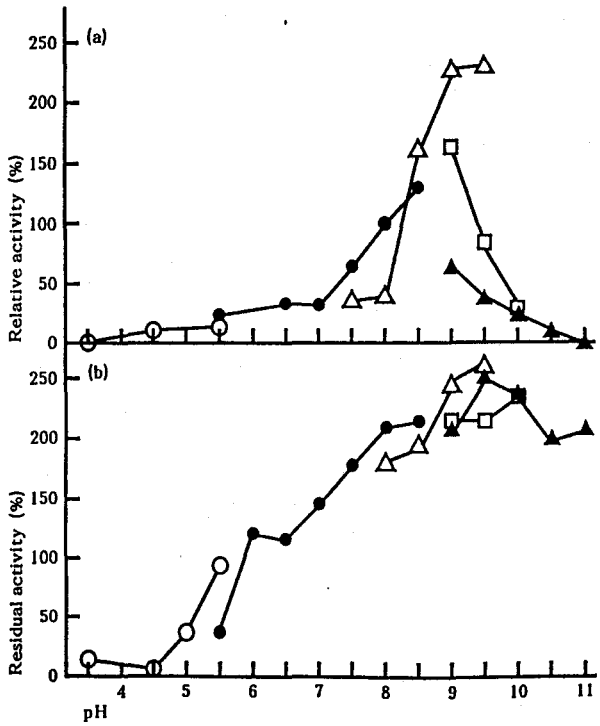

Figure 4-16. Effects of pH on (a) *the activity of the* E. coli *enzyme and* (b) *its stability.*

(a) *Optimum pH.* The assay mixture (with 0.0057 U of activity/ml) was incubated under the standard conditions except for the use of different buffers. The activity is expressed relative to that at pH 8.0 with phosphate.

(b) *pH stability.* The enzyme (with 0.0040 U of activity) was incubated at 37℃ for 6 hr in 0.5 ml of the indicated buffer (0.025 mmol). Then 0.2 mmol of the standard buffer and 3 μmol of cytosine in 0.5 ml of water were added (final volume, 1.0 ml), and the assay was done for 30 min. The activity that remained is expressed relative to that of the untreated enzyme.

○, Sodium acetate; ●, potassium phosphate; △, Tris-HCl; □, glycine; ▲, borate.

Common inhibitors. Some possible enzyme inhibitors were tested (table 4-9b). *p*-Chloromercuribenzoic, *p*-chloromercuriphenylsulphonic, and mersaryl acids inhibited the reaction strongly, suggesting again that the enzyme is an SH-enzyme [11].

Effects of amino acids. Many amino acids were examined to see if they affected the enzyme activity (table 4-9c). Some amino acids, including L-aspartic acid, L-phenylalanine, and L-serine, seemed to accelerate enzyme activity slightly.

4.3. DISCUSSION

A large amount of this enzyme is necessary if it is to be used in therapy. Cultural conditions for enzyme production were investigated. Citric acid was a suitable carbon source. The pH of the culture increased during cultivation, and growth and enzyme activity increased when the pH was controlled so as not to exceed a certain value. Control of the culture pH at around 8.5 with citric acid was most effective for enzyme production (table 4-3). So, more citric acid was added to the culture by a pH-stat constructed for this purpose. Large-scale production of the enzyme was then possible.

Many affinity adsorbents were prepared and tested with a crude extract of the bacteria. One group of adsorbents were NA-Sepharoses. As the amino group of cytosine is the site of attack by the enzyme, affinity chromatography on carriers with terminal amino groups was examined. There is another possibility: that NA-Sepharoses themselves act as affinity adsorbents, or rather as hydrophobic adsorbents, in many cases [67]. So, this group of gels was tried. Many kinds of NA$_n$-Sepharoses with an *n* of up to 12 were synthesized and used in column chromatography of the crude enzyme solution (table 4-4). Some of the gels gave good recovery, although with little purification.

The second group of adsorbents tried were mercaptopyrimidines immobilized on Sepharose *via* a hexyl spacer, by use of a procedure for the immobilization of adenosine 5'-monophosphate [17]. The ligands were coupled by replacement reactions with 1,6-diaminohexane, which was to be the spacer, and further coupled with CNBr-activated Sepharose *via* the resulting amino termini. Chromatography on columns of the gels prepared from 2-mercaptopyrimidine and 2-thiobarbituric acid purified the enzyme effectively

Table 4-9. Effects of various classes of common enzyme effectors on the activity of E. coli *cytosine deaminase.*

Enzyme (0.020 U) was incubated for 30 min in 1.0 ml of the standard assay mixture containing 1 or 0.1 mM of the substance being tested as an effector. Activity is expressed relative to that of the control (with no tested as an effector. Activity is expressed relative to that of the control (with no supplements).

Compound tested	Relative activity	
	1 mM	0.1 mM
(a) *Metal salts*		
$AgNO_3$	0	0.2
$BaCl_2$	1.0	
$CaCl_2$	1.1	
$Cd(CH_3COO)_2$	0.9	
$CoCl_2$	0.9	
$CuSO_4$	0	0
$FeCl_2$	0.6	
$FeCl_3$	1.0	
$Hg(CH_3COO)$	0	0
$HgCl_2$	0	0
KCl	0.9	
$MgCl_2$	0.9	
$MnCl_2$	1.1	
NaCl	0.9	
$NiCl_2$	0.8	
$PbCl_2$	0.8	
$SnCl_2$	0.7	
EDTA[a]	1.0	
(b) *Possible inhibitors*		
NaF	1.1	
NaN_3	1.0	
$NaNO_2$	0.9	
Na_2HAsO_4	1.0	
α,α'-Dipyridyl	0.9	
o-Phenanthroline	0.4	
2-Mercaptoethanol	0.9	
N-Ethylmaleimide	1.0	
p-Chloromercuribenzoic acid	0	0
p-Chloromercuriphenylsulfonic acid	0	0
Mersalyl acid[b]	0	0
Iodoacetic acid	1.1	

(c) *Amino acids*	
L-Alanine	1.1
D-Alanine	1.2
β-Alanine	1.1
L-Arginine	1.0
L-Asparagine	1.0
D-Asparagine	0.9
L-Aspartic acid	1.3
D-Aspartic acid	0.9
L-Cysteine	1.0
L-Cystine	0.9
L-Glutamic acid	1.2
D-Glutamic acid	0.7
L-Glutamine	1.3
Glycine	1.3
L-Histidine	1.2
L-Hydroxyproline	1.2
L-Isoleucine	1.3
DL-Isoleucine	1.3
L-Leucine	1.2
L-Lysine	1.0
L-Methionine	1.1
DL-Methionine	1.4
L-Ornithine	1.2
L-Phenylalanine	1.4
L-Proline	1.1
L-Serine	1.4
L-Threonine	1.2
D-Threonine	1.2
L-Valine	1.2
DL-Homoserine	1.1
DL-Norvaline	1.0
DL-α-Amino-*n*-butyric acid	1.4

[a] Ethylenediaminetetraacetic acid tetrasodium salt (as chelator); [b] salyrganic acid, *i.e.*, *O*-[(3-[hydroxymercuri]-2-methoxypropyl)carbamoyl]-phenoxyacetic acid.

(table 4-5). A column of the former gel was used to demonstrate an example of effective elution with a linear gradient of salt (figure 4-7) and the same gal was used in the purification experiments (figure 4-8).

The third and fourth groups of adsorbents were carboxy- and aminopyrimidines immobilized on NA_6- and CA_6-Sepharose, respectively, by carbodiimide coupling. Both adsorbents have C_6 spacers. Chromatography of the crude enzyme solution with the immobilized carboxypyrimidines, shown in figure 4-3, gave worse results than the above NA_n-Sepharoses, although these carboxypyrimidines were coupled with NA_6-Sepharose. Most aminopyrimidines were more effective, giving better recovery, than the above immobilized carboxypyrimidines. Immobilized 5-aminouracil was used in one of the steps in the purification experiments (figure 4-9). Completeness of the coupling was not analysed in any gel, but the ligands seemed to be attached to the carrier gel because the gels adsorbed the enzyme activity; CA_6-Sepharose, with which these amino ligands were coupled, did not.

The length of the spacers affects both the coupling of the ligand to the carrier and the affinity of the enzyme to the immobilized ligand. Adsorption of the enzyme to affinity gels of various spacer lengths was studied with use of isoorotic and orotic acids as carboxy ligands and 2-aminopyrimidine and 5-aminouracil as amino ligands. For the carboxy ligands, the length of the spacers was varied by the use of NA_n-Sepharoses with various n numbers prepared in the same way as the first group of adsorbents. NA_n-Sepharoses were reacted with the carboxy residues of the ligands by carbodiimide coupling, giving a fifth group of adsorbents. Columns of the gels prepared from the ligands with C_4 and C_{12} spacers gave good purification (figure 4-4). It is not known why the chromatography resulted in a poor yield when the spacer was C_5 to C_8 long, or why there was no elution with C_9 or C_{10}, these lengths being between those of the C_4 and C_{12} spacers. On the other hand, for the amino ligands, the use of various ω-aminoalkanoic acids was not appropriate because such acids (other than 6-aminohexanoic acid) are usually not commercially available. So a plan to construct long spacers with carboxy termini was made. That is, α,ω-dicarboxy-alkanes m carbon atoms long were linked to NA_n-Sepharoses by cardodiimide coupling to give CA_m-NA_n-Sepharoses. The carbodiimide reaction again coupled the carboxy termini to the amino residues of the ligands, and the resulting conjugates formed a sixth group of adsorbents. The spacers constructed were not polyhydrocarbons, but they contained amido linkages, and were therefore were somewhat hydrophilic, which was expected to give good results.

In fact, the results were poor (figure 4-5). Why enzyme activity was not eluted from the carriers with CA_{10}-NA_8-spacer is not known. Under different conditions, elution might occur, because the poor recovery from the carriers with CA_{10}-NA_6-,CA_6-NA_8-,and CA_8-NA_8-spacers might mean that the enzyme had been bound to the affinity gels too tightly. Compared with the results for the two ligands immobilized on CA_6-Sepharose (figure 4-4), this class of spacers was not effective.

As usual, gradient elution and fractionation into small fractions resulted in good purification with many of the adsorbents prepared and used here; a typical example with a good effect was demonstrated with the immobilized 2-mercaptopyrimidine (table 4-5). Out of the 68 kinds of adsorbents described here, this one and another, 5-aminouracil immobilized on CA_6-Sepharose, were used in the purification experiment (figure 4-8,9). Both absorbents, however, gave little purification, probably because the enzyme had been already partially purified from the crude cell extract of *E. coli*.

The main objective of this study was to obtain enough of this thermostable enzyme to use clinically in the treatment of cancer. The effect of heat on enzyme activity was examined here. The effects of incubation beforehand at 37℃ were observed (figure 4-15a). Activity was stable up to 55℃ when incubation was for 6 hr (figure 4-15b). The activity, which was stable in the cell extract, was still stable after it was purified to homogeneity. This stability was useful for the purification of the enzyme by heat treatment in this study. As was shown in chapter 2, when the enzyme is encapsulated in a semi-permeable cellulose tube and left at 37℃ in buffered saline for a fortnight, or implanted under the skin of rats for a month, the biological half-life was about 10 days. So, this enzyme was very stable to heat, but not stable enough for therapeutic use.

Enzyme activity was optimum at around pH 9. The enzyme was active at the region of physiological pH, which is promising for the clinical use of this enzyme.

The enzyme was inhibited by very low concentrations of Ag^+, Cu^{2+}, Hg^+, or Hg^{2+}, and by *p*-chloromercuribenzoic, *p*-chloromercuriphenylsulphonic, or mersaryl acid. These findings strongly suggested that this enzyme was an SH-enzyme [11].

A partially purified enzyme was encapsulated in a semi-permeable membrane in Chapter 2. The intact enzymic activity of the capsule was proportional to the substrate concentration; for example, it was about 0.003 U toward 5FC at the concentration of 0.3 mM with a capsule enclosing 0.31

U of activity. According to calculation, this meant that the activity inside the capsule, 0.31 U, was sufficient for therapy.

As mentioned before, two bacterial cytosine deaminases were purified to homogeneity from *Serratia marcescens* and *Pseudomonas aureofaciens*, and their kinetic properties were studied [63,64,82,83]. This enzyme was also purified from *Salmonella typhimurium*; it was extremely thermostable, but deaminated 5FC less than it did cytosine [76]. This enzyme was also purified from bakers' yeast, and its characteristics was studied [36,80]. I further purified this enzyme from compressed yeast and studied it later, as will be described in the next chapter. The enzyme from *E. coli* was different from the three enzymes from the other bacteria and the enzyme from yeast in many respects. Discussion and comparison of results will be presented in Chapter 5.

4.4. EXPERIMENTAL

Unless otherwise noted, all experiments were done as already described.

4.4.1. *Materials*

Chemicals. 2-Mercaptopyrimidine was obtained from Nacalai Tesque, Inc. (Kyoto). 5-Aminouracil and mersalyl acid were from the Sigma Chemical Co. (St. Louis).

Other materials. Sepharose 4B, Sephacryl S-300, Blue Dextran 2000, and LKB Ampholine carrier ampholyte (pH 3.5 ~ 10; no. 1809-101) were obtained from Pharmacia. The disk ultramembrane was UK-10 (molecular-weight cutoff of 10,000), fitted in UHP-25 holders, both from Toyo Roshi. The hollow-fibre membrane filter cartridge used was Labo-module ACL-1050 (cutoff, 13,000) from Asahi Chemical Industry (Osaka) or Amicon H1P10 (cutoff, 10,000) from Grace Japan KK (Tokyo). The calibration proteins for gel permeation chromatography were in a commercial kit (Combithek 104558) obtained from Boehringer Mannheim Yamanouchi KK (Tokyo) and bovine thyroglobulin from Sigma. The calibration proteins for isoelectric focusing were from a commercial pI marker kit that was obtained from the Oriental Yeast Co. (Tokyo) and contained various kinds of acetylated cytochrome *c* with different pI values.

Instruments. The pH electrode was of a combination type (no. 6028-10T) from Horiba Ltd. (Kyoto). The pH meter, titrator, and titrigraph were models 28, 11, and SBR 2c, respectively, from Radiometer A/S (Copenhagen). The peristaltic pump was model SJ-1220 from the Atto Corp. (Tokyo). The mini-jar fermentor was of the 2-L type (model M-100) from the Tokyo Rikakikai Co. (Tokyo). The jar fermentor with three 30-L vessels (model MSJ-30L-3) was manufactured by the B.E. Marubishi Co. (Tokyo), and was equipped with pH controllers. The glass-bead mill was a Dyno-mill KDL, manufactured by Willy A. Bachofen Maschinenfabrik (Basel).

4.4.2. *Microorganism and culture conditions*

E. coli K-12 IFO 3301 was used. The optimum medium for the production of the enzyme was adapted from the growth medium that was already described as described in the first half of this chapter, and was composed of 1.04% citric acid, 3% peptone, 0.2% yeast extract, 1% KH_2PO_4, 0.3% NaCl, and 0.01% $MgCl_2$; it was adjusted to pH 7.0 with an NaOH solution. The organism was cultivated in growth medium at 30°C on a shaker in test tubes (16 × 165 mm), each containing 5 ml of medium, or else in 500-ml shaking flasks, each containing 100 ml of growth medium.

pH-stat culture. A pH controller was constructed with a pH electrode, a pH meter, a titrator, and a titrigraph, with a peristaltic pump to inject acid. When pH-stat culture was done in a mini-jar fermentor, the controller was connected to the fermentor. The amount of acid added to a unit volume of the culture over a certain time was calculated from the amount of acid added to the whole culture through the pH controller (read from the titrigraph) divided by the volume of the working culture (calculated), and added to make the sum of acid added so far to the unit volume.

Tank culture. A 2-L mini-jar fermentor containing 1.5 L of the optimum medium, or a 3 × 30-L triple jar fermentor

equipped with pH controllers and with 25 L of the growth medium in each vessel was used. Culture was done of a 1% inoculum from the seed flask at 30℃ with aeration (about half the working-culture volume/min) and agitation (500 rpm in the mini-jar and 200 in the jar fermentor). An antifoam agent was used when necessary. The pH was kept at 8.5 after it reached that value with 50% citric acid solution added by the pH-stat.

4.4.3. *Crude enzyme solution*

The cells were collected after 24 hr of culture and disrupted by ultrasonic oscillation to give the cell extract.

Cells in the tank were harvested by centrifugation. After being washed they were disintegrated in a glass-bead mill in a 6-dl continuous grinding chamber containing about 5 dl of 0.3-mm glass beads. The chamber and reservoir were cooled to below 10℃ with circulating refrigerant. Disruption was done in the continuous circulating operation mode and monitored by occasional inspection of the cell suspension under a microscope. Milling was stopped when almost all cells were disrupted. The cell suspension in the chamber, the reservoir, and the washings of the glass beads were combined, centrifuged to remove cell debris, concentrated in a hollow-fibre ultrafilter (Labo-module) under reduced pressure, and dialysed by overnight diafiltration in the same filter in a similar way. The dialysed solution is referred to as cell extracts.

For use in the affinity chromatography experiments, this extract was further fractionated by precipition with ammonium sulphate between 30 and 55%. The precipitate was dissolved in and dialysed overnight against the buffer. The resulting dialysed solution, referred to as the crude enzyme solution, was used here.

4.4.4. *Enzyme and protein assays*

Spectrophotometric method. The activity of cytosine deaminase was usually assayed by the spectrophotometric method (described in section 2.4.4).

Ammonia method. To study substrate specificity, deaminating activity toward the pyrimidine compounds tested was assayed by discontinuous assay of ammonia formed in the reaction with a commercial assay kit, Determiner NH_3, comprising a glutamate dehydrogenase system and $NADPH_2$ (Kyowa Medex Co., Tokyo).
glutamate dehydrogenase system and $NADPH_2$ (Kyowa Medex Co., Tokyo; Determiner NH_3).

Disk-plate assay of the enzyme. On column chromatography of the enzyme in the purification steps, the activity was located in the eluted fractions by the disk-plate assay described in Chapter 3.

Protein assay. For convenience, OD_{280} was used instead of protein concentration in some cases. (Thus, the specific activity is expressed in U/mg protein, or else in U/ml per OD_{280}).

4.4.5. *Immobilization of pyrimidines on Sepharose*

Sepharose 4B was used (called Sepharose here). Its volume was the packed volume.

4.4.5.1. *CNBr-activated Sepharose*

Sepharose was activated with CNBr by a method that makes use of acetonitrile [51]. Washing must be completed within 90 sec. Then the coupling was done immediately. The coupling mixture was left in the cold for 2 days to allow masking of any unreacted active groups on the gel.

4.4.5.2. *CA- and NA-Sepharoses*

To construct reactable spacer arms on the Sepharose gel matrix, ω-aminoalkanoic acid or α,ω-diaminoalkane that was n carbon atoms long was attached to CNBr-activated Sepharose as described above. ω-Aminoalkanoic acid or α,ω-diaminoalkane (2 mmol) was dissolved in the coupling buffer (20 ml) and allowed to react with the activated Sepharose (10 ml). The resulting gel was ω-carboxyalkylimino-Sepharose or ω-aminoalkylimino-Sepharose, and is referred to here as CA- or NA-Sepharose. The length of the spacer is expressed as the number of carbon atoms of the spacer, in the form of CA_n- or NA_n-Sepharoses (*e.g.*, CA_6-Sepharose for 5-carboxypentylimino-Sepharose, and NA_8-Sepharose for 8-aminooctylimino-Sepharose). After being washed, the resulting gel was suspended in water or buffer to the final volume of 20 ml.

4.4.5.3 *Carbodiimide coupling*

Compounds with carboxy and amino groups were immobilized on NA- and CA-Sepharoses, respectively, by the usual carbodiimide coupling between amino and carboxy groups. Each of these compounds (20 μmol) was dissolved in water (1.8 ml), and to this, the suspension of NA- or CA-Sepharose (10 ml) was added for coupling. The mixture was agitated gently, and occasionally adjusted to pH 4.5 with dilute HCl or NaOH, and to this, a solution (0.20 ml; pH 4.5) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (10 mg) was added. The mixture was agitated gently, and occasionally adjusted to pH 4.5 with dilute NaOH. Coupling was overnight at room temperature. The gel was washed thoroughly with water on a sintered-glass filter.

4.4.5.4. *Immobilization of mercaptopyrimidines*

1,6-Diaminohexane was attached to mercaptopyrimidines by the replacement of the thiol groups of the mercaptopyrimidines with the amino group of 1,6-diaminohexane [17]. The derivatives obtained, with amino-terminated side-chains, were

1,6-Diaminohexane was attached to mercaptopyrimidines by the replacement of the thiol groups of the mercaptopyrimidines with the amino group of 1,6-diaminohexane [17]. The derivatives obtained, with amino-terminated side-chains, were then coupled with CNBr-activated Sepharose. That is, a solution (1.0 ml) containing 1,6-diaminohexane (4.0 mmol) and one of the mercaptopyrimidines (40 μmol) was put in a test tube (15 × 105 mm); the tube was sealed with a Teflon-lined screw cap, placed in an oven horizontally (because the reac-

tion was surface-dependent), and heated at the temperature and for the period decided in preliminary experiments (80～140℃, 6～16 hr). After being cooled, the solution was transferred to an evaporator flask with water (50 ml), and evaporated to a heavy concentrate under reduced pressure. This was diluted with 10 ml of water and lyophilized several times to remove excess 1,6-diaminohexane. The final residue, which was free from the smell of 1,6-diaminohexane, was dissolved in 0.2 M sodium bicarbonate buffer (pH 9.5; 20 ml) and used in the coupling reaction with CNBr-activated Sepharose without further purification or analysis of the complete reaction.

4.4.5.5. *Affinity adsorbents for enzyme purification*

Two affinity adsorbents were prepared on a larger scale than described above by the same methods.

2-Mercaptopyrimidine (0.40 mmol) and 1,6-diamonohexane (40 mmol) were dissolved in 10 ml of water, divided into 10 aliquots in test tubes, and heated at 80℃ for 6 hr for coupling. The conjugate was purified and again conjugated to 100 ml of the CNBr-activated Sepharose. 5-Aminouracil (0.2 mmol) was immobilized on CA_6-Sepharose (100 ml) by carbodiimide coupling.

4.4.6. *Gel permeation*

Before the gel-permeation chromatography, the enzyme solution was concentrated by ultrafiltration first in a hollow-fibre membrane filter (Amicon) by circulation under pressure, and then with a disk ultramembrane under nitrogen gas pressure. Chromatography was done with a constant flow (15 ml/hr) of the dialysis buffer (as described in section 4.2.3.5), and 1-ml fractions were collected. Elution was monitored by UV absorption at 280 nm.

For estimation of the molecular weight of the enzyme, the column was calibrated with several marker proteins. Blue Dextran 2000 was used as a void volume marker (molecular weight, 2,000,000). For every run, two or three of the markers (1 mg each) were dissolved in 2 ml of the buffer and used.

4.4.7. *Electrophoresis*

In all of the electrophoreses below, polyacrylamide gels were polymerized in glass tubes (5φ × 65 mm). After the run, the gels were taken out, set in a mixture of 3.5% sulphosalicylic acid and 10% trichloroacetic acid in 30% methanol for fixation of protein, stained with 0.01% Coomassie brilliant blue G-250, destained thoroughly with several changes of 7% acetic acid in 25% ethanol, and stored and photographed in 7% acetic acid in 10-mm test tubes.

Disc gel electrophoresis. Discontinuous polyacrylamide gel electrophoresis was done by Davis' method [19] in 5% gel with a constant current of 2～4 mA per gel rod.

SDS-gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) was done by the method of Weber and Osborn [75] in 5% gel with the usual amount of cross-linker at 8 mA per gel rod.

Isoelectrofocusing. Analytical isoelectric focusing was done by Wrigley's method [78] in columns for disc electrophoresis containing gels made of 5% polyacrylamide and 2% carrier ampholyte (pH 3.5～10). The anode and cathode solutions were 0.02 M H_3PO_4 and 1 M NaOH, respectively.

CHAPTER 5

Cytosine deaminase from bakers' yeast [e]

Cytosine deaminase was extracted from commercial compressed bakers' yeast and purified to an almost homogeneous state. The enzyme activity was more than 200 U/mg of protein, which was several times higher than reported before. The molecular weight was 41,000 by gel permeation. The pI was at pH 4.7. 5FC, 5-methylcytosine, and creatinine were other substrates for the enzyme. An experiment with inhibitors suggested that the enzyme was an SH-enzyme. The enzyme was unstable to heat, with a biological half-life of about 0.5 hr at 37℃. Characteristics of the enzyme, especially its substrate specificity, were compared with those reported earlier for other cytosine deaminases from bacteria and a mould.

5.1. INTRODUCTION

Cytosine deaminase from *Escherichia coli* cannot yet be used in the local chemotherapy of cancer proposed in Chapter 2. First, it is difficult to culture the bacteria on a large scale in order to obtain enough activity. As often occurs during the purification of enzymes, *E. coli* cytosine deaminase was obtained only in low yield when highly purified, as described in Chapter 4. Second, because of possible pyrogenic reactions, it is preferable to use a highly purified preparation of the enzyme or else to use an enzyme from other, presumably safer sources than bacteria.

Cytosine deaminase can, however, be obtained in large quantities from commercial compressed bakers' yeast, as described in Chapter 1. Yeast has been used as an agent of alcoholic fermentation from ancient times, and also as an agent for bread leavening in the Western world; so, it seems to be a relatively safe source. As described in Chapter 1, yeast cytosine deaminase deaminates 5-methylcytosine (5MC), a 5-substituted cytosine. It also deaminates 5FC, another 5-substituted cytosine, in *Saccharomyces cerevisiae* [27]. So, the cytosine deaminase of bakers' yeast (*S. cerevisiae*) should convert 5FC to 5FU, and could be used in place of *E. coli* cytosine deaminase. Although the yeast enzyme is unstable to heat (at 37.5℃) [48], which would prevent its use in long-term therapy in the body, it might be stabilized by immobilization or other techniques.

An extract from compressed yeast deaminated 5FC to form 5FU in a preliminary experiment, so the enzyme from the yeast was used in the following studies.

The enzyme from commercial bakers' yeast has been purified, 340-fold in a first report [36], and "completely" in a second report [80], although the result was purification by only severalfold from the first preparation. This chapter describes the further purification of this enzyme and its characteristics, especially its substrate specificity in comparison with those of other origins.

5.2. RESULTS

5.2.1. *Purification of yeast cytosine deaminase*

Cytosine deaminase was extracted from compressed yeast by plasmolysis. The enzyme was purified from the extract by fractional precipitation with ammonium sulphate, anion-exchange chromatography (with DEAE-cellulose or DEAE-Sephacel), hydrophobic chromatography (with Octyl-Sepharose CL-4B), and gel-permeation chromatography (with Toyopearl HW-50S). A typical procedure was as follows, and the results are summarized in table 5-1.

5.2.1.1. *Cell extract*

Compressed yeast (2.0 kg) was broken into flakes with the fingers, mixed with ethyl acetate (200 ml) with a spatula,

Table 5-1. Purification of cytosine deaminase from bakers' yeast.

Step	Protein (mg)	Protein (OD_{280} × ml)	Activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell extract	98,000		3,600	0.037	1	100
Ammonium sulphate 1	39,00		3,000	0.077	2.1	82
Ammonium sulphate 2	11,000		1,500	0.14	12	42
DEAE-Sephacel	78		420	5.5	150	12
Octyl-Sepharose CL-4B	4.9	6.1	210	42	1,100	5.7
Toyopearl HW-50S	0.41[a]	0.51	160	250[a]	6,800[a]	2.9

[a] The amount of protein was calculated with the assumption that the concentration was proportional to the OD_{280}.

stirred for 30 min with a magnetic stirrer, and further stirred for a few hours after the addition of 2.0 L of buffer that had been saturated 15% with ammonium sulphate and adjusted to pH 7.0, so that the cells were plasmolysed. To the autolysate obtained, 8.0 L of buffer was added; the mixture was left in a cold room and stirred for a few minutes daily. Cell debris was removed by centrifugation after 3 days. The clarified lysate (11 L) was referred to as the cell extract.

5.2.1.2. *Ammonium sulphate fractionation*

The extract was brought to 70% saturation with ammonium sulphate. The precipitate that formed overnight was collected by centrifugation, dissolved in 3.5 L of buffer, and dialysed thoroughly against the buffer. The dialysed solution (4.0 L) was fractionally precipitated with ammonium sulphate from 50 to 73% saturation. The precipitate was collected with 1.0 L of 20 mM buffer, dialysed as above, and diluted to 4.0 L with the same buffer. Fractionation was again done between 56 and 70% saturation, and the precipicipitate was dissolved in 350 ml of 10 mM buffer, followed by dialysis.

5.2.1.3. *Anion-exchange chromatography*

The above fraction (350 ml) was put onto a column (4.4ϕ × 14 cm) of DEAE-Sephacel previously equilibrated with buffer. The column was washed thoroughly with buffer, and the enzyme was chromatographed with a linear gradient of potassium chloride from 0 to 100 mM in 1.0 L of buffer at a flow rate of about 50 ml/hr. Active fractions were collected and combined (40 ml).

5.2.1.4. *Hydrophobic chromatography*

The above 40 ml was dialysed against 3.1 M ammonium sulphate buffered at pH 7 with 100 mM potassium phosphate, centrifuged to remove the precipitates formed, and put onto a column (2.2ϕ × 12.5 cm) of Octyl-Sepharose CL-4B. Elution was done with a linear gradient of ammonium sulphate from 3.1 to 0 M in 200 ml of 100 mM buffer at a flow rate of about 25 ml/hr. The active fractions were combined (18 ml), concentrated by ultrafiltration to about 1.5 ml, and diafiltrated with 50 mM buffer (pH 7.4) containing 100 mM

potassium chloride.

5.2.1.5. *Gel-permeation chromatography*

The above dialysed solution was chromatographed on a column (1.3ϕ × 63 cm) of Toyopearl HW-50S with the same buffer as above at a flow rate of 14 ml/hr. The active fractions were collected (8 ml; 130 U; absorbance of 0.130 at 280 nm), concentrated by ultrafiltration to about 1.4 ml, and passed again through the same column in the same way, but with a recycling operation of a total of two passages. The chromatography was monitored with a UV-recorder. The active fractions were combined (12 ml). They contained 100 U of enzyme activity, corresponding to a yield of 2.9%, and had the absorbance of 0.043 at 280 nm, from which it was calculated that there was 0.41 mg of protein, with the assumption that absorbance and protein concentration were proportional.

5.2.1.6. *Purity of the enzyme*

The fraction above had a specific activity of over 200 U/mg (table 5-1). This corresponds to a 6800-fold purification over the cell extract, as calculated from the purification factors from step to step. When the gel was stained, discontinuous gel electrophoresis gave one dense main band and two very

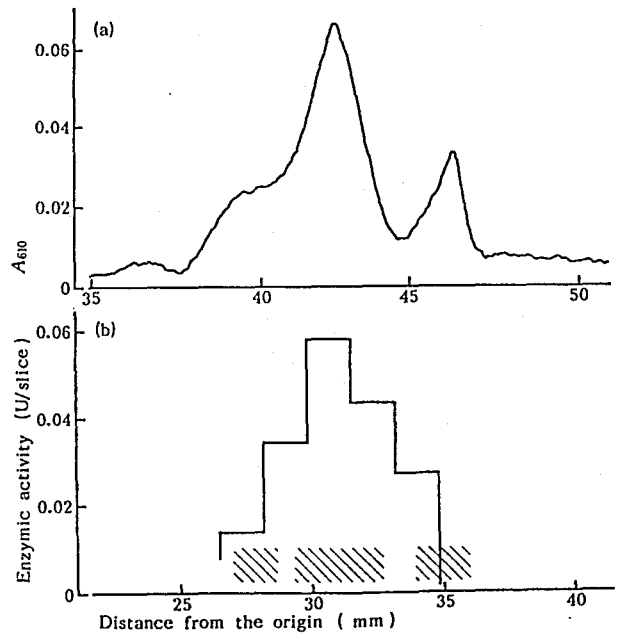

Figure 5-1. Disc polyacrylamide gel electrophoresis of the purified cytosine deaminase.
Details are in the text.
(a) Dye-staining. A densitogram is shown.
(b) Activity-staining. The histogram shows the activity. The approximate position of the stained band is shaded.

faint bands on both sides of the main band. Figure 5-1a shows a densitogram. To identify which of the three bands was the enzyme protein, another gel rod was cut lengthwise into two almost equal parts. One piece was studied by dye-staining, and the other was studied by activity-staining, as follows. The second piece was further cut crosswise into 1.7-mm slices. Every slice was transferred into a test tube, ground with a microspatula, and extracted with 1 ml of buffer. This extract was assayed for enzyme activity. The main band accompanied the enzyme activity (figure 5-1b).

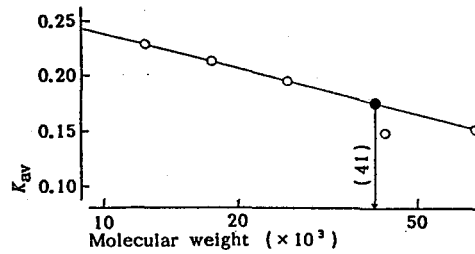

Figure 5-2. Graphic estimation of the molecular weight of yeast cytosine deaminase by recycling gel-permeation chromatography on Toyopearl HW-50S.

Details are in the text.

Standard proteins (molecular weights): horse heart cytochrome *c* (12,500); myoglobin (17,000); α-chymotrypsinogen A (25,000); ovalbumin (43,000); bovine serum albumin (68,000). Blue Dextran 2000 (2,000,000) and uracil (130) were used as a void volume and an end-point marker, respectively.

○, Cytosine deaminase; ●, standard proteins.

5.2.2. *Properties of yeast cytosine deaminase*

The final fraction above was examined as follows for certain properties.

5.2.2.1. *Molecular weight*

The column used in the fractionation for gel-permeation chromatography was calibrated with standard proteins, and the molecular weight of the enzyme protein was found from a graph to be 41,000 (figure 5-2).

5.2.2.2. *Isoelectric point*

Isofocusing electrophoresis of the enzyme gave the isoelectric point of pH 4.7 (figure 5-3).

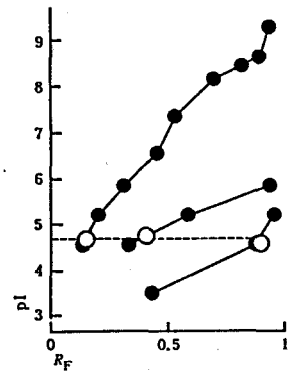

Figure 5-3. Graphic estimation of pI of yeast cytosine deaminase by isofocusing electrophoresis.

Details are given in the experimental section. The data from three runs are plotted.

○, Cytosine deaminase; ●, pI markers.

5.2.2.3. *Substrate specificity*

Besides cytosine and 5FC, the enzyme deaminated 5MC and creatinine. The ratio of the activities toward these substrates at the concentration of 3 mM was 1 : 0.8 : 0.8 : 0.3.

5.2.2.4. *Michaelis constants*

The effects of the concentration of cytosine or 5FC as substrate were examined. From Lineweaver and Burk double-reciprocal plots, the apparent Michaelis constants, K_m, was

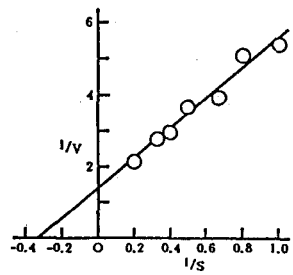

Figure 5-4. Effects of substrate concentration on yeast cytosine deaminase.

A Lineweaver-Burk plot is shown.

calculated to be 3.1 and 1.2 mM, respectively (figure 5-4).

5.2.2.5. *Other properties*

In the following experiments, partially purified enzyme preparations were used. These consisted mainly of the fraction that was eluted after the main fraction in the hydrophobic chromatography step, and they had the specific activity of about 10 U/mg of protein, which was about 4 % of the highest purity appearing in this study.

5.2.2.5.1. *Effects of temperature*

The enzyme was incubated under the standard conditions but at various temperatures. The optimum temperature for the reaction was at around 30℃ when the pH was 8.0, and at around 40℃ when the pH was 7.0 (figure 5-5a). When the enzyme was incubated at various temperatures for 15 min, activity decreased at over 30℃ (figure 5-5b). After 30 min of incubation at 37℃, the enzyme retained about half of its activity.

5.2.2.5.2. *Effects of pH*

The activity of the enzyme at different pHs was studied (figure 5-6a). The optimum for the reaction was at around pH 7.5. The effect of pH on the stability of the enzyme was examined by measurement of the activity after incubation at different pHs at 37℃ for 15 min (figure 5-6b). It seemed that the activity was most stable at around pH 7.5.

5.2.2.5.3. *Effectors*

Some metal salts that may be general inhibitors for enzymes and some amino acids were tested to see if they affected the reaction with this enzyme from bakers' yeast (table 5-2). The enzyme was inhibited strongly by low concentrations (0.1 and 1 mM) of Ag^{+}, Hg^{+}, and Hg^{2+}, and weakly by high concentrations (1 mM) of Fe^{2+}, Fe^{3+}, and Pb^{2+}, suggesting that this enzyme was an SH-enzyme [11], like the enzyme from *E. coli* described in Chapter 4. *p*-Chloromercuribenzoic, *p*-chloromercuriphenylsulphonic, and mersalyl acid at 1- and 0.1-mM concentrations strongly inhibited the reaction, and *o*-phenanthroline at 1 mM did so weakly, suggesting again that the enzyme was an SH-enzyme [11]. The 36 amino acids, including five D- and DL-isomers, did not affect the enzyme activity much (table 5-2c).

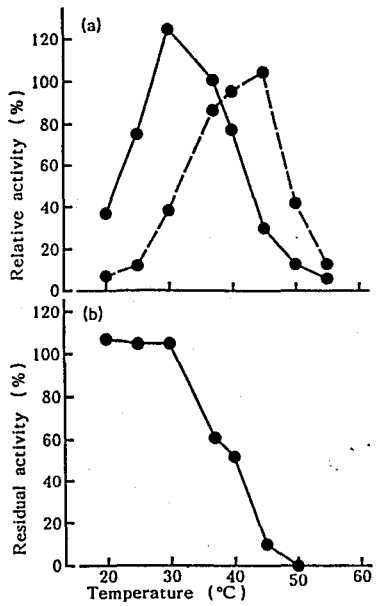

Figure 5-5. Effects of heat on (a) *the activity of yeast cytosine deaminase and* (b) *its stability.*

(a) *Optimum temperature.* The enzyme with 0.016 U of activity in 0.5 ml of the reaction mixture was incubated under the standard conditions except for temperature and pH. The activity (mean of two measurements) is expressed relative to that of the control (37℃; pH 8.0). ——, pH 8.0; – – –, pH 7.0.

(b) *Thermostability.* The enzyme with 0.011 U of activity was incubated at the indicated temperature for 15 min in 0.25 ml of 40 mM phosphate buffer (pH 7.4). The mixture was immediately cooled in an ice bath, and 0.15 ml of 0.67 M phosphate buffer (pH 8.0) was added. After several minutes of incubation at 37℃, the assay reaction was started by the addition of 0.10 ml of 15 mM cytosine solution that had been incubated, for total volume of 1.0 ml. The activity that remained is expressed relative to that of the untreated sample assayed at pH 8.0 with 0.20 M phosphate. ●, Phosphate (pH 8.0); ○, borate (pH 9.5).

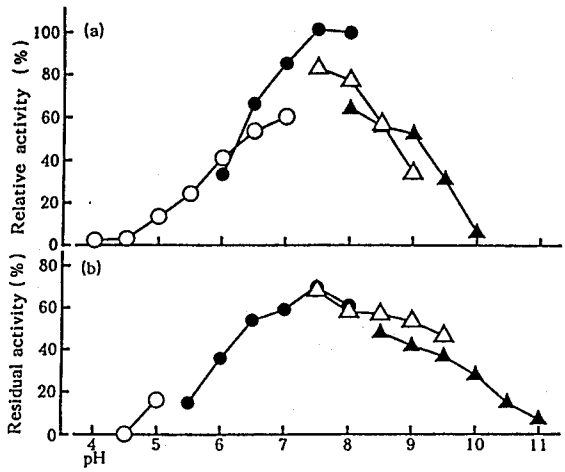

Figure 5-6. Effects of pH on (a) *the activity of yeast cytosine deaminase and* (b) *its stability.*

(a) *Optimum pH.* The assay mixture with 0.017 U of activity/ml was incubated under the standard conditions except for the different buffers at 50 mM. The activity (mean of two to four measurements) is expressed relative to that at pH 8.0 with 50 mM phosphate.

(b) *pH stability.* The enzyme with 0.012 U of activity was incubated at 37℃ for 15 min in 0.20 ml of the indicated buffer at the final concentration of 50 mM. Then 0.10 mmol of phosphate buffer, pH 8.0, and 3μ mol of cytosine in 0.30 ml of water were added (final volume, 0.50 ml), and the assay was done as usual except that the reaction was for 60 min. The residual activity (mean of two or three measurements) is expressed relative to the activity of the untreated enzyme.

○, Sodium acetate; ●, potassium phosphate; △, Tris-HCl; ▲, sodium borate.

5.2.3. *Substrate specificity of the other cytosine deaminases*

Cytosine deaminases from *Serratia* [63] and *Pseudomonas* [64] were prepared and examined for activity toward 5FC and creatinine, which were not used before as the substrates, in comparison with their activity toward cytosine and 5MC (table 5-3). *E. coli* cytosine deaminase was again examined as well, together with cytosine deaminases from some of the other strains of *E. coli*; all K-12 strains or their derivatives. These enzymes from *S. marcescens* and *P. aureofaciens* deaminated both 5FC and creatinine. Cytosine deaminase from *E. coli* K-12 IFO 3301 deaminated 5MC, as described in Chapter 4, but all of these enzymes from the other strains of *E. coli* did not. The enzymes from HB101 and C600 deaminated neither 5FC nor 5MC; the enzyme from HB101 deaminated creatinine, and that from C600 did not.

Table 5-2. Effects of various classes of common enzyme effectors on the activity of yeast cytosine deaminase.

Enzyme (0.016 U) was incubated for 60 min in 1.0 ml of the standard assay mixture containing 1 or 0.1 mM of the substance being tested as an effector. Activity is expressed relative to that of the control (with no supplements).

Compound tested	Relative activity	
	1 mM	0.1 mM
(a) *Metal salts*		
$AgNO_3$	0	
$BaCl_2$	1.0	
$CaCl_2$	0.9	
$CdCl_2$	0.9	
$CoCl_2$	0.9	
$CuSO_4$	1.0	
$FeCl_2$	0.7	
$FeCl_3$	0.9	
$Hg(CH_3COO)$	0	0.1
$HgCl_2$	0	0
KCl	0.8	
$MgCl_2$	0.9	
$MnCl_2$	1.0	
NaCl	1.0	
$NiCl_2$	0.8	
$SnCl_2$	0.9	
EDTA	0.8	
(b) *Possible inhibitors*		
NaF	1.1	
NaN_3	1.2	
$NaNO_2$	1.2	
Na_2HAsO_4	1.1	
α,α'-Dipyridyl	1.1	
2-Mercaptoethanol	1.1	
N-Ethylmaleimide	1.0	
p-Chloromercuribenzoic acid	0	0
p-Chloromercuriphenylsulfonic acid	0	0
Mersalyl acid	0	0
Iodoacetic acid	1.1	
(c) *Amino acids*		
L-Alanine	1.2	
D-Alanine	1.0	
β-Alanine	1.1	
L-Arginine	1.2	
L-Asparagine	1.1	
L-Aspartic acid	1.1	
D-Aspartic acid	1.1	
L-Cysteine	1.2	
L-Cystine	1.0	
L-Glutamic acid	1.1	
D-Glutamic acid	1.1	
L-Glutamine	1.1	
Glycine	1.1	
L-Histidine	1.1	
L-Hydroxyproline	1.1	
L-Isoleucine	1.0	
DL-Isoleucine	0.9	
L-Leucine	1.1	
L-Lysine	1.0	
L-Methionine	1.1	
D-Methionine	1.1	
L-Ornithine	1.1	
L-Phenylalanine	1.0	
L-Proline	1.0	
L-Serine	1.0	
D-Serine	1.0	
L-Threonine	1.1	
DL-Threonine	1.0	
L-Tryptophan	1.2	
D-Tryptophan	1.3	
L-Tyrosine	1.0	
L-Valine	1.0	
DL-Homoserine	1.1	
DL-Norvaline	1.0	
DL-α-Amino-*n*-butyric acid	1.0	
β-Amino-*n*-butyric butyric acid	1.1	

Table 5-3. Substrate specificity of cytosine deaminases from various bacteria.

A bacterial species was cultured on a shaker at 30°C in 3~10 flasks each containing 1 dl of the indicated medium. Cells were collected and made into cell-free extract. Cytosine deaminase was purified, when so indicated.

Strain	Culture medium[a]	Crude cell extract		Purification procedure[c]	Activity (mU/mg) towards:			
		Activity (U/L[b])	Protein (g/L[b])		Cytosine	5FC	5MC	Creatinine
E. coli								
K-12 IFO 3301[d]	E	2.0	7.7	A, D	27	28	5	10
K-12 IFO 3301[e]	E	5.0	5.8	A, D	60	6	0	7
K-12 AKU 0005[f]	E	0.4	0.2	None	1.8	2.6	0.0	1.2
HB 101	E	5.8	6.6	A, D	15	0	0	4
C600	E	0.20	0.35	A, D	3.0	0	0	0
P. aureofaciens								
IFO 3521	P	9.0	2.6	A	3.4	1.2	0.8	0.6
S. marcescens								
IFO 3054	E	1.9	13	A	0.8	0.1	0	0.3
AKU 0063	E	0.7	7.7	A	9.0	5.1	0	4.6
S. polymuthicum								
AKU 0062	E	0	?	None	0	0	0	0

[a] Medium E was used in [63] and Chapter 2; medium P was used in [64]. [b] Activity and protein obtained per L of culture are shown. [c] Procedure A was ammonium sulphate fractional precipitation; D was DEAE-cellulose column chromatography. Both were used in [63,64] and Chapter 4. [d] This IFO strain had been stocked in the laboratory for many years and was used in the experiments in Chapter 1. [e] This was here newly obtained from the IFO. [f] This was IFO 3208 and has been stocked in the AKU for decades.

5.3. DISCUSSION

The almost pure cytosine deaminase obtained was the purest preparation from bakers' yeast reported so far. The enzyme from bakers' yeast was previously reported to be purified partially [36] or completely [80], so the purification of the enzyme was started from autolysates of the yeast, following the reported procedures. The enzyme soon reached the same purity (specific activity) as these preparations reported to be highly or completely purified. However, the present preparation was still very impure at that stage by the criterion of disc gel electrophoresis. Then, as described here, this enzyme was purified 6800-fold, to the specific activity of over 200 U/mg of protein, which is probably severalfold higher than that of the "pure" enzyme already reported [80]. The present enzyme was different from the other(s) [36,80] in many of its properties.

The enzyme seemed to be an SH-enzyme. The enzyme activity was as labile (figure 5-5b) as when the enzyme was a crude or partially purified preparation [48]. This would

cause difficulties if it were used in long-term cancer chemotherapy. It must be stabilized by immobilization or the like before use. In fact, it could be much stabilized by immobilization on a variety of adsorbents (Chapter 6).

Five cytosine deaminases have been purified in this laboratory: this one from yeast, one from the mould *Aspergillus fumigatus* IFO 5840 [84], and three from bacteria: *E. coli* K-12 IFO 3301 described in Chapter 4, *Serratia marcescens* IFO 3054 [63,82], and *Pseudomonas aureofaciens* IFO 3521 [64,83]. These are listed in table 5-4. They were different from each other in molecular weight, substrate specificity, and thermal stability. Cell extracts from many bacteria have been also examined for enzyme activities involved in the metabolism of cytidine; some half of the extracts had cytosine deaminase activity [65]. About half of the cytosine deaminases are thermostable, and the others unstable [63]. The substrate specificity of this enzyme toward cytosine and 5MC in cell extracts from many bacteria has been studied, together with the activity of the other enzymes concerned in the metabolism of cytidine [65]. In that study, these enzymes were classified according to substrate specificity and heat stability as the *Serratia* type, which is cytosine-specific and thermostable, and the *Pseudomonas* type, which is non-specific and unstable. The *Escherichia* enzyme dealt with in Chapter 4 was classified as the *Serratia* type [83], but here I would like to char-

Table 5-4. Characteristics of cytosine deaminases from various origins.

Cytosine deaminases from *Serratia* and *Pseudomonas* were examined. This enzyme from some strains of *E. coli* was also examined as described in the discussion section.

Organism	Cytosine deaminase activity (U/mg)	Substrate specificity[a] C : F : M : Cr	Molecular weight ($\times 10^3$) Whole[b]	Molecular weight ($\times 10^3$) Sub-units[c]	*p*I (pH)	Thermal resistance[d]	pH optimum[e]	Inhibitors[f]	Reference
Bakers' yeast (Oriental Yeast Co.)	250	100 : 70 : 70 : 30	41		4.7	30℃ (15 min)	7.5	*p*CMB, M^+	Ch. 5
Aspergillus fumigatus IFO 5840	11	100 : 40 : 30 : -	32				7	*p*CMB, M^+	[84]
Escherichia coli K-12 IFO 3301	9.8	100 : 70 : 10 : -	200	35; 46	5.7	55℃ (6 hr)	9	*p*CMB, M^+	Ch. 2, 4
Serratia marcescens IFO 3054	2.4	100 : - : 0 : -	580	72		50℃ (10 min)		dGMP	[63]
Pseudomonas aureofaciens IFO 3521	1.2	100 : - : 20 : -	630	45		40℃ (5 min)	10	*p*CMB, M^+	[64]
Salmonella typhimurium	17	100 : 5 : 0 : -	230	54		50℃ (10 min)	10	M^+	[76]
Flavobacterium filamentosum	20	100 : 30 : 4 : 180	270	44					[24]
Alkaligenes denitrificans ssp. *denitrificans*									[41]
Fraction I	14	100 : 120 : - : 0	200						
Fraction II	4	100 : 120 : - : 0	37						
Arthrobacter sp. J11			37						[41]

[a] Percentage ratio of cytosine, 5FC, 5MC, and creatinine values. -, not measured. [b] Determined by gel-permeation chromatography; or by [c] SDS-disc electrophoresis. [d] Temperature is shown at up to which the enzyme was stable after incubation for a certain period. [e] At the optimum temperature. [f] M^+ means metal ion(s). [g] *A. denitrificans* ssp. *denitrificans*.

acterize this as a third type, called the *Escherichia* type, because its activity against 5MC is unlike that of the *Serratia* type and because it is thermostable.

In addition to the purified enzymes mentioned above, cytosine deaminases of *Salmonella typhimurium* [76], *Flavobacterium filamentosum* (as creatinine deiminase) [23], and strains *Arthrobacter* sp. JH-13 (extracellular) [79], *Arthrobacter* sp. J11 [41], and *Alcaligenes denitrificans* ssp. *denitrificans* J9 (containing two enzymes) [41] have been studied in detail (table 5-4). The outstanding feature of yeast and mould cytosine deaminases is the molecular weight of the protein, which is about 40,000 and 30,000, respectively, unlike those of the bacterial enzymes reported, which are 200,000~600,000, as described in Chapter 4 and in other reports [23, 63, 64, 79].

The substrate specificity of cytosine deaminases dealt with here is summarized in table 5-5. The yeast cytosine deaminase deaminated 5MC, 5FC, and creatinine, as well as cytosine. 5MC was also deaminated by other cytosine deaminases, from bakers' yeasts [27,48], *Aspergillus* [84], *Pseudomonas* [64], and the *Escherichia* described in Chapter 4, but not by the cytosine deaminase from *Serratia* [63] or *Salmonella* [76]. Moreover, it inhibits the activity in *Flavobacterium* toward cytosine [23]. The conversion of 5FC to 5FU by cytosine deaminase in fungi [27,39] explains the antifungal activity of 5FC. 5FC is also deaminated by other cytosine deaminases, those from *Aspergillus* [84], *Salmonella* [76], *Escherichia* (described in Chapter 4), *Flavobacterium* [23], two strains of *Arthrobacter* [41,79], *Pseudomonas* (table 5-3), *Alcaligenes* with two enzymes [41], and many other bacteria [38]. All of the tested cytosine deaminases deaminated or deiminated creatinine (table 5-3), which can be called creatinine deiminase activity, as do most of the other cytosine deaminases reported, with rare exceptions [23,41]. *E. coli* K-12 cytosine deaminase deaminates 5MC and 5FC as described in Chapter 4, as well as cytosine and creatinine (table 5-3). In an early report, 5MC and 5-hydroxymethylcytosine were found to be inert to the cytosine deaminase of this bacterium [15]. So *E. coli* cytosine deaminase has been believed not to deaminate 5-substituted cytosines. In fact, as shown in table 5-3, cytosine deaminases from two derivative strains of *E. coli* K-12, HB101 and C600, did not deaminate 5FC or 5MC at all, and the activities of cytosine deaminase toward 5MC and 5FC in comparison to the cytosine values varied from strain to strain in some stock strains of *E. coli* K-12 that had been preserved in different laboratories. This subject still remains complicated.

Table 5-5. Summary of substrate specificity of cytosine deaminases from various origins.

Organism	Relative activity towards:			
	Cytosine	5FC	5MC	Creatinine
E. coli K-12				
IFO 3301 (Ch. 2)	100	70	10	-
IFO 3301[a]	100	100	20	40
IFO 3301[b]	100	10	0	10
AKU 0005[c]	100	140	0	70
(HB 101)	100	0	0	30
(C600)	100	0	0	0
S. marcescens				
IFO 3054[d]	100	-	0	-
IFO 3054[b]	100	10	0	40
AKU 0063	100	6	0	50
S. polymuthicum	0	0	0	0
P. aureofaciens				
IFO 3521[d]	100	-	20	-
IFO 3521[b]	100	40	30	20
S. typhimurium [d]	100	5	0	-
F. filamentosum [d]	100	30	4	180
A. denitrificans [d,e] (both enzymes)	100	120	-	0
Arthrobacter sp. J11[d]	100	-	-	-
Bakers' yeast (Ch. 5)	100	70	70	30
A. fumigatus [d]	100	40	30	-

[a] Stock of this laboratory. [b,c] Newly supplied from the [b] IFO and [c] AKU. [d] Cited data. [e] *A. denitrificans* ssp. *denitrificans*.

5.4. EXPERIMENTAL

Unless otherwise noted, all experiments were done as already described.

5.4.1. *Materials*

Materials. Compressed bakers' yeast was obtained from the Oriental Yeast. DEAE-Sephacel, Octyl-Sepharose CL-6B, and the pI calibration kit of marker proteins for isoelectrofocusing (kit no. 17-0471-01) were obtained from Pharmacia. Myoglobin and haemoglobin were from Sigma. Toyopearl HW-50S was from the Tosoh Corp. (Tokyo).

Instruments. The UV monitor was M&S-Altex 150B UV-recorder (M&S Instruments, Inc., Osaka). The densitometer was model 39433 of Gelman Sciences Japan, Ltd. (Tokyo).

5.4.2. *Enzyme assay*

The enzyme activity was assayed spectrophotometrically as described in section 2.4.4; the reaction was for 30 min because of the instability of the activity to heat.

For the study of the substrate specificity, the substrates and products in the assay mixtures were assayed by dual-wavelength spectrophotometry as described in section 2.4.5: cytosine and uracil at both 250 and 280 nm; 5FC and 5FU, or 5MC and thymine, at 255 and 290 nm. The creatinine specificity was estimated by enzymatic assay of the ammonia that formed and by comparison with the cytosine value obtained by the same method for reference.

5.4.3. *Ultrafiltration and diafiltration*

Concentration and diafiltration of a small volume of enzyme solution were done with a Toyo Roshi ultramembrane.

5.4.4. *Electrophoreses*

Densitograms for the stained disc gels were recorded at the wavelength of 610 nm. Ampholine carrier ampholytes were in the pH ranges of 3~10 and 4~6. Cytosine deaminase on the electrophoresis gels was located by the activity-staining described in section 5.2.1.6; the gels were cut in 2-mm slices.

CHAPTER 6

Stabilization of cytosine deaminase from bakers' yeast by immobilization [f,g]

The purpose of the study described in this chapter was to stabilize the yeast enzyme, which is very labile, as described in Chapter 5, for long-term use in the local cancer chemotherapy that was proposed in Chapter 2.

The enzyme was stabilized through immobilization by various techniques. When the enzyme was immobilized on Eupergit C, a commercial epoxy-acrylic resin, the highest yield of apparent enzymic activity was about 80% of the original activity. The enzymic activity decayed exponentially at 37℃ over a few weeks, the biological half-life being about 10 days. Other commercial adsorbents also resulted in good stability but yields were lower: half-life of enzymic activity was 15 days for Formyl-Cellulofine and 24 days for CNBr-activated Sepharose 4B; yields were 20~67 and 21%, respectively.

The Eupergit-C-immobilized enzyme was further encapsulated in cellulose tubing for dialysis or else entrapped in urethane polymer. The intact cellulose capsules had 4% of activity of the immobilized enzyme contained inside the tubing. The enzyme capsules thus made were stable, with a half-life of activity of about 5 months when the polyurethane capsules had been incubated for 4 months at 37℃.

6.1. INTRODUCTION

Local chemotherapy for cancer may be possible with the combined use of 5FC given orally and cytosine deaminase implanted locally, as described in Chapter 2. Cytosine deaminase is easily obtained from bakers' yeast. However, it is unstable to heat when it is crude or partially purified [48]. If proteolytic activity contaminating the crude enzyme solution is involved in this denaturation, the decrease in the activity might be overcome by purification of the enzyme to a high level. In fact, because the enzyme is still unstable after purification (Chapter 5), it may be intrinsically labile, and not usable for long-term therapy without immobilization, which usually brings about stabilization of enzymes. So, I decided to try to immobilize it in order to stabilize it.

The enzyme was immobilized on many carriers by various relatively simple techniques, and tested for stability at 37℃. Some stable forms of the immobilized enzyme were obtained. The yields of enzymic activity and the life-spans of the activity will be described in this chapter.

The immobilized enzyme might be encapsulated for therapy, in cellulose membrane as described in Chapter 2, or in polyurethane, which is easily handled and which has biocompatibility with animal tissues (for review, see [25]). Some characteristics of such enzyme capsules made in this

way will be also described here.

6.2. RESULTS

6.2.1. *Immobilization of cytosine deaminase*

Many lots of bakers' yeast cytosine deaminase, with various levels of activity and purity, were immobilized on many carriers by a number of methods, as follows (figure 6-1). The results are summarized and listed in table 6-1.

6.2.1.1. *Immobilization of the enzyme on Sepharose 4B through CNBr-activation*

Sepharose 4B was activated with cyanogen bromide (CNBr)

Table 6-1. Summary of immobilization of bakers' yeast cytosine deaminase.
Arrows and their directions indicate variations in the conditions (ascending) and the results (ascending and descending).

Carrier and method	No. of lots made	Enzyme preparation used			Immobilized cytosine deaminase		
		Specific activity (U/mg)	Activity (U/g wet gel[a])	Protein (mg/g wet gel[a])	Activity (U/g wet gel[a])	Yield (%)	Half-life[b] (days)
Sepharose 4B, activated with CNBr/CH_3CN	27	n.m.[c]	3.6	—	0~1.4	0~41	32
Sepharose 4B, activated with CNBr/$(C_2H_5)_3N$ (0.06↗0.48 mmol of CNBr used/g gel)	8	13	20	1.5	3.6±0.2	18±1	6.4
CNBr-activated Sepharose 4B (commercial)	1	2.5	5.9	2.3	1.23	21	24
Eupergit A	4	n.m.[c]	4.0	—	1.7±0.2	42±5	8.9
Eupergit C	3	10	3.1↗12	0.30↗1.2	0.45↗1.7	14±1	n.m.[c]
	6	110	8.4↗130	0.079↗1.3	7.3↗27	86↘20	9.6
	7	160	4.4↗140	0.026↗0.85	4.1↗26	96↘18	n.m.[c]
Formyl-Cellulofine	10	10	2.6↗85	0.25↗8.2	1.6↗16	59↘22	15
Urethane prepolymer PU-3 (0.33 g of PU-3 used/g gel)	7	4.9	0.15↗0.61	0.031↗0.12	0.029↗0.085	19↘14	1.6
(0.15↗0.67 g of PU-3 used/g gel)	10	4.9	0.31	0.06	0.11↗0.24	30↗80	n.m.[c]

[a] The weight of the completed gel. [b] Half-life of the decrease in enzymic activity at 37°C. [c] Not measured.

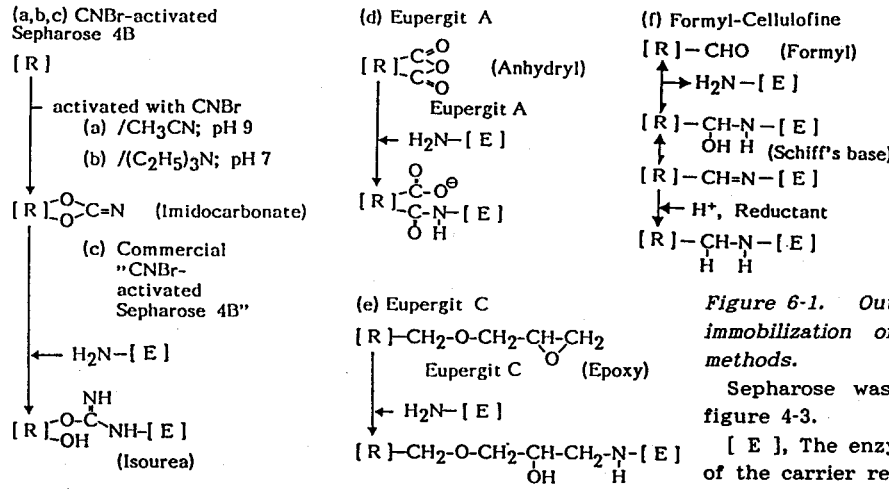

Figure 6-1. Outline of procedures for immobilization of proteins by different methods.

Sepharose was activated as shown in figure 4-3.

[E], The enzyme molecule; [R], matrix of the carrier resin.

by simplified methods involving the use of acetonitrile and an alkaline pH with carbonate buffers (the acetonitrile method; figure 6-1a), which was already used in Chapter 4, to immobilize pyrimidine compounds for affinity chromatography of the *Escherichia coli* enzyme, or the use of triethylamine and a neutral pH in aqueous acetone (the triethylamine method; figure 6-1b). A ready-to-use adsorbent was also used (figure 6-1c).

6.2.1.1.1. *Acetonitrile method*

Sepharose 4B, in 27 lots of 5 g (wet) each, was activated in an alkaline buffer with different amounts (50～200 μl/g of gel) of a CNBr/acetonitrile solution (100 g and 50 ml, each) for 0.5～2.0 min. The enzyme was immediately brought in contact with the activated gel. An enzyme solution, with 3.6 U of activity in 2.0 ml of the coupling buffer, was used per gram of gel. The reproducibility of the method was poor and the conditions showed no correlation with the yield of activity, which was from 0～41% (or 14±12%; n=27), compared to the original activity; five lots gave a 0% yield, and the other nine lots gave yields of less than 10%. Eight lots with yields of 22% or more (30±7%) were chosen from among the 27 lots. Together they contained 44 U of enzymic activity (about 9% of the total activity used in all lots).

6.2.1.1.2. *Triethylamine method*

Sepharose 4B, in 26 lots of 5 g (wet) each, was activated by the triethylamine method; the concentration of CNBr used was from 2.5 to 480 μmol/g of gel. The activated gel was then coupled with 1.5 mg of the protein (with 2.0 U of activity) per gram of gel. When plotted on a log scales (figure 6-2), the

amount of cyanate ester that formed on the gel, which is an index of the coupling capacity, was linearly correlated (with a slope of 1.01; $r=0.98$; $n=20$) with the amount of CNBr used (2.5~60 μmol/g of gel). The maximum was 23 μmol of ester/g of gel. The activity immobilized on the gel was also linearly correlated (with a slope of 0.89; $r=0.96$; $n=12$) with the amount of CNBr used (up to 15 μmol/g of gel). The maximum was 3.6 ± 0.2 U/g of gel (18 ± 1% yield; $n=8$), when 60 μmol or more of CNBr was used. On non-log scales (not shown), the amount of cyanate ester formed was proportional (with a factor of 0.14 ± 0.02; $n=14$) to the amount of CNBr used (up to 15 μmol/g of gel); the activity was also proportional (with a factor of 0.22 ± 0.04; $n=12$) to the amount of CNBr used (up to 15 μmol/g of gel). These indicated that the amount of the gel used was sufficient to immobilize the amount of protein used here, and that 15 μmol of CNBr was sufficient to activate 1 g of the gel. The reproducibility of this method was good, unlike in the acetonitrile method.

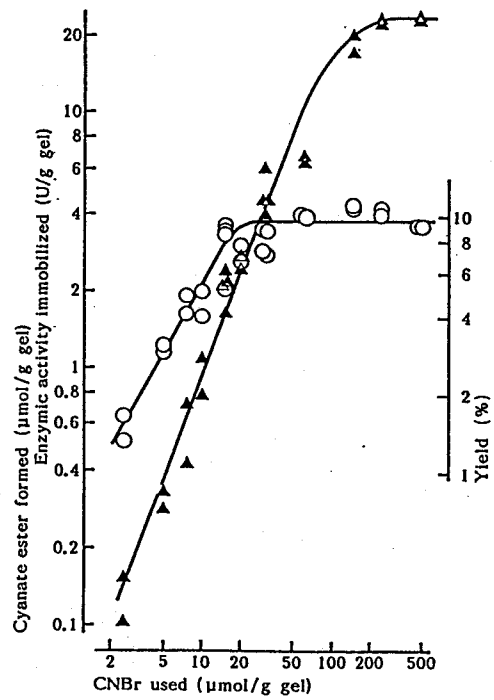

Figure 6-2. Immobilization of yeast cytosine deaminase on Sepharose by the triethylamine method.

▲, Cyanate ester formed; O, activity of the immobilized enzyme and yield of immobilization.

6.2.1.1.3. *Ready-to-use carrier*

For convenience, commercial CNBr-activated Sepharose 4B was also used. An enzyme solution containing 2.1 U of activity and 0.82 mg of protein in 0.2 ml was brought to the volume of 0.7 ml with 0.1 M sodium bicarbonate (pH 8.3)/0.5 M sodium chloride, and left overnight in a refrigerator. To block unreacted active residues, the gel was removed under reduced pressure with the use of filter paper, transferred into 0.2 M Tris buffer (pH 7.0), and placed in the cold for 2 days. After being thoroughly washed, the immobilized enzyme weighed 0.35 g (packed and wet) and contained 0.15 U of activity, a yield of 21% of the initial activity.

6.2.1.2. *Immobilization of the enzyme on Eupergit A and C*

Two more commercial ready-to-use carriers, which have different mechanisms from the mechanism of CNBr activation, were also used. They were acrylic beads, Eupergit A and C, with the active residues being anhydride and epoxy (oxirane) groups, respectively, constructed on the resin matrices (figure 6-1d,e).

Eupergit A. A solution of the enzyme containing 8.7 U of activity was brought to 4 ml with the addition of potassium phosphate buffer (pH 7.0; final concentration, 0.8 M) and mixed with 1.0 g of dry Eupergit A gel. The immobilized enzyme obtained weighed about 2.2 g and contained 3.7 ± 0.5 U of activity, a 42 ± 5% yield ($n=4$).

Eupergit C. An enzyme solution with the specific activity of 10 U/mg of protein was diluted with 1 M potassium phosphate buffer, pH 7.4, so that 1.0 ml contained 9～37 U, and was poured evenly over 0.25 g of dry Eupergit C gel. The completed gels, weighing 0.76 g wet, were assayed for enzymic activity (figure 6-3,△). The yield was 14±1% (n=3), which was poor, but very reproducible. In the same way, two other enzyme solutions with higher specific activities were diluted and mixed with Eupergit C (figure 6-3,○,●). The yield was 20～80% of the activity used; it was highest when the lower range of enzyme concentrations was used. The apparent Michaelis constants, *Km*, of the immobilized cytosine deaminase were about 30 mM for cytosine and about 4 mM for 5FC.

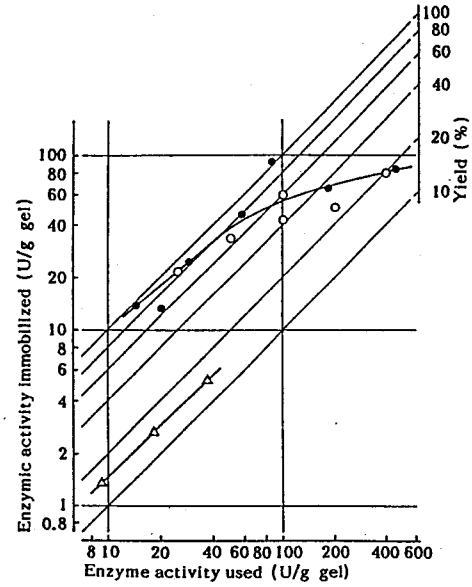

Figure 6-3. Immobilization of yeast cytosine deaminase on Eupergit C.

Enzyme preparations used had specific activities, in U/mg, of 10 (△), 110 (○), and 160 (●).

Thin diagonal lines and the right ordinate give conversion from the yield of enzymic activity to the yield percentage over the activity used.

6.2.1.3. *Immobilization of the enzyme on Formyl-Cellulofine*

Formyl-Cellulofine forms Schiff's base on contact with amino residues of the protein to be bound (figure 6-1f). Coupling was completed by the addition of sodium cyanoborohydride ($NaCNBH_3$), a reductant for the fixation of Schiff's base.

An enzyme solution with the specific activity of 10 U/mg of protein was diluted with the coupling buffer so that 2.0 ml contained 0.9～30 U, and reacted with 1.0 g of wet Formyl-Cellulofine. The completed gels were assayed for enzymic activity. The activity yielded was plotted against the activity on a log scale (figure 6-4). The plot gave a straight line, and the result was very reproducible (r=0.99; n=10). With a small amount of protein, the yield was high.

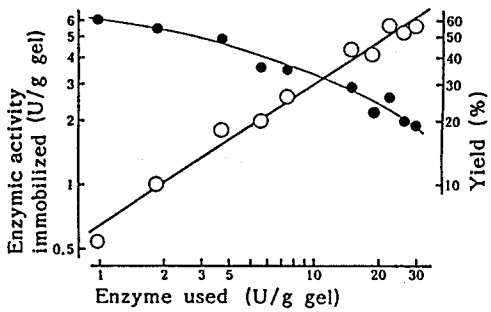

Figure 6-4. Immobilization of yeast cytosine deaminase on Formyl-Cellulofine.

○: activity of the immobilized enzyme; ●: yield of immobilization.

6.2.1.4. *Immobilization of the enzyme with urethane prepolymer*

Urethane prepolymer entraps any kind of material when it polymerizes. Polymerization occurs through intermolecular condensation between isocyanate residues of the prepolymer, forming a urea linkage with the liberation of carbon dioxide gas and giving a wet polymer like urethane foam; the reaction starts with the attack by a water molecule on an isocyanate residue.

An enzyme solution with a specific activity of 4.9 U/mg of protein was reacted with the PU-3 urethane prepolymer. The standard conditions were as follows. The enzyme solution was diluted so as to contain 0.92 U (0.19 mg) in 2.0 ml of buffered saline, added to 1.0 g of PU-3, and mixed to cause polymerization, giving 3.0 g of a polymer. This was cut into

small cubes and assayed for enzymic activity. The conditions for polymerization were examined with the use of combinations of 0.46～1.8 U of activity and 0.45～2.0 g of prepolymer to give 3 g of gel, and by partial replacement of PU-3 with PU-6. Results, when plotted on a log scale, showed that correlation of the amount of enzyme used and of the amount of prepolymer used (up to 1.5 g) with the enzymic activity in the completed gel (figure 6-5). The standard conditions above gave enzymic activity of 0.16±0.04 U/g of gel (yield of 53±13%; $n=2$). From the plot, the reading was 0.17 U/g. The slopes were 0.49 and 0.56 for the enzyme and the prepolymer, respectively ($r=0.93, 0.90$; $n=7, 10$). The ratio of PU-6 to PU-3 had no effect (not shown).

The 11 most active gels, which contained 0.073±0.014 U of activity/g of gel, were selected from among the above 25 lots without PU-6 and combined. Samples were incubated at 37℃. Enzymic activity was assayed after 1 and 5 days, and found to be 0.046±0.020 and 0.010±0.006 U/g of gel, respectively. Thus, the half-life was 1.6 days ($r=-0.87$); activity certainly remained, but not much, or else it soon disappeared.

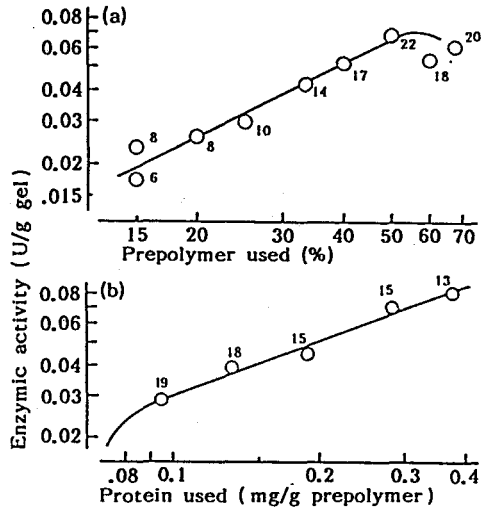

Figure 6-5. Immobilization of yeast cytosine deaminase with urethane prepolymer PU-3.
Small numerals are the yield percentage of immobilization.

6.2.2. *Stability of the immobilization products*

The immobilized enzyme preparations thus made were examined mainly for their thermal stability. These were incubated in buffered saline at higher temperatures than usual in the accelerated stability test [14] or for longer periods than before.

6.2.2.1. *Sterilization of the immobilized enzyme*

Some immobilized enzyme preparations were treated with sodium azide, Osvan (benzalkonium chloride), or mercuric chloride. Sodium azide did not affect the activity of the enzyme immobilized on Eupergit C or Sepharose 4B activated with CNBr by the acetonitrile method, unlike Osvan or mercuric chloride, the second of which completely destroyed the activity of the latter immobilized enzyme preparation.

6.2.2.2. *Thermal stability of the immobilized enzymes*

Accelerated stability test. Some of the immobilized enzyme preparations were tested for thermal stability as follows.

They were suspended in buffered saline in screw-capped test tubes, and incubated at 37, 50, 60, or 75℃ for several hours. At certain times, the enzymic activity remaining in the tubes was assayed (figure 6-6). After incubation for 4 hr, the immobilized enzyme preparations tested did not decrease in enzymic activity at 37℃; the activity decreased slowly at 50 and 60℃, and rapidly at 75℃. After 4 hr at 60℃, about 15% of the activity of the enzyme immobilized on Eupergit C remained. This immobilized enzyme preparation and that on Sepharose 4B activated with CNBr by the acetonitrile method completely lost their activity within 1 hr of incubation at 75℃. But by the triethylamine method, about 10% of the initial activity was retained after 1 or 2 hr, although the gel was deformed by the heat.

Stability at 37℃. Thermal stability at 37℃ of some of the immobilized enzymes prepared here was examined again as follows, with monitoring of the activity remaining for several weeks. Portions of sterile gels were transferred to screw-capped test tubes with buffered saline, which were placed in an incubator at 37℃ for a few weeks. At intervals, they were taken out and assayed for enzymic activity by incubation with added substrate. Some examples are shown in figure 6-7; semi-logarithmic plots for the decrease in enzymic activity were used for the calculation of the half-lives, listed in the legend to the figure.

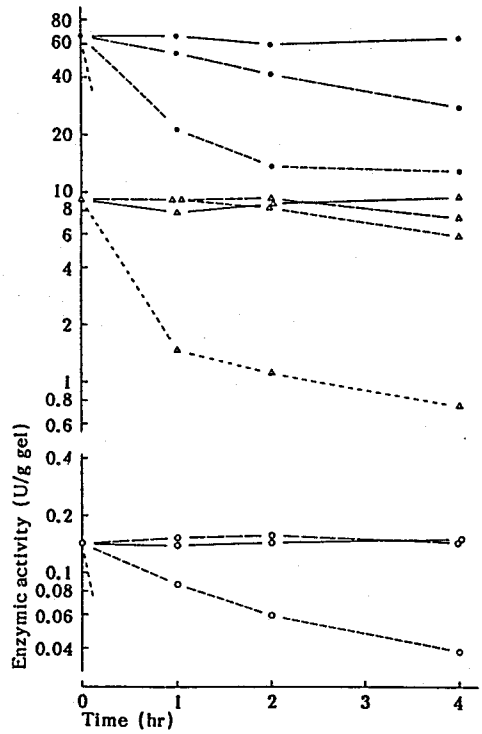

Figure 6-6. Accelerated stability test of the immobilization products from yeast cytosine deaminase.

Cytosine deaminase immobilized on Sepharose 4B activated with CNBr by the acetonitrile method (○) or the triethylamine method (△) or else immobilized with Eupergit C (●) was incubated at 37℃ (———), 50℃ (— —— —), 60℃ (— — —), or 75℃ (— — —).

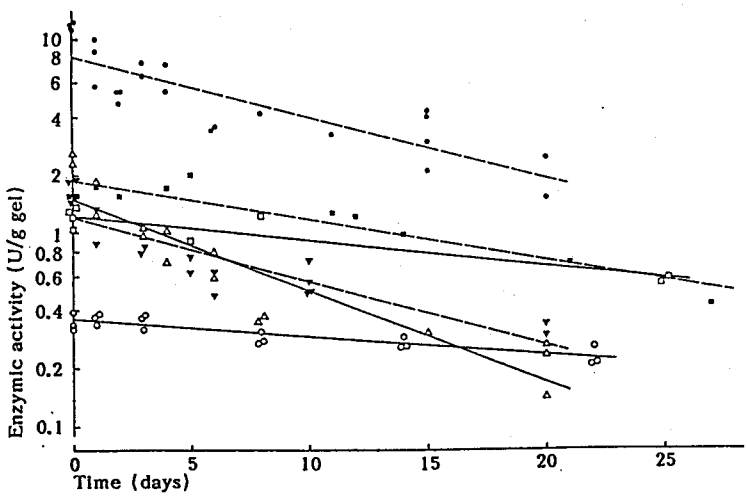

Figure 6-7. Stability of immobilized cytosine deaminase at 37℃.

○, △, □, Immobilized on Sepharose 4B activated with CNBr by the acetonitrile method or the triethylamine method, or with the pre-activated carrier, respectively; ▼, Eupergit A; ●, Eupergit C; ■, Formyl-Cellulofine.

Gel (symbol)	Half-life (days)	*r*	*n*
○	32	-0.90	18
△	6.4	-0.92	16
□	24	-0.92	8
▼	8.9	-0.88	18
●	9.6	-0.85	23
■	15	-0.94	11

6.2.3. *Long-term stability of the Eupergit-C-immobilized enzyme*

The enzyme solution was mixed with dry Eupergit C as described before (section 6.2.1.2). Two lots of the resultant wet gel contained about one-half of the initial activity used (table 6-2).

Table 6-2. Immobilization of cytosine deaminase on Eupergit C.

Gel	Enzyme solution used (ml)	Completed gel (wet)		Yield (%)
		Weight (g)	Enzymic activity (U/g gel)	
●	1.0	3.3	24	44
○	2.5	3.3	65	46

6.2.3.1. In vitro *stability of the immobilized enzyme*

The above immobilized enzyme was sterilized with azide as described before (section 6.2.2.1), suspended in phosphate-buffered saline in vials, which were sealed tightly, and incubated at 37℃ for 1 month. At times, portions were withdrawn and the enzymic activity remaining was assayed. Activity decreased exponentially to about a quarter (○) or a half (●) of the initial activity (figure 6-8, left). With longer incubation up to 8 months, activity did not decrease much further (figure 6-8, right). The half-lives for the above two

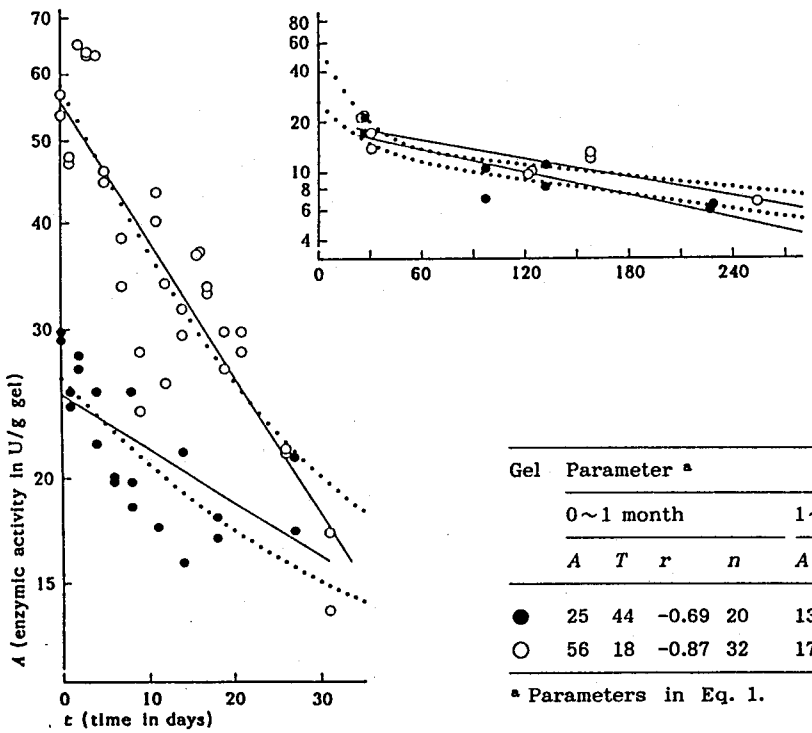

Figure 6-8. Stability at 37℃ of yeast cytosine deaminase immobilized on Eupergit C.
Two lots of the immobilized enzyme (symbols ● and ○ in table 6-2) were used. Parameters for the decline were obtained by linear regression for periods of 0~1 and 1~8 months (solid lines) or by least-squares calculation by the use of equation 1 for the whole period, 0~8 months (dotted lines), and given below.

Gel	Parameter [a]													
	0~1 month				1~8 months				0~8 months through					
	A	*T*	*r*	*n*	*A*	*T*	*r*	*n*	A_1	T_1	A_2	T_2	*r*	*n*
●	25	44	-0.69	20	13	140	-0.84	8	13	14	14	210	0.95	26
○	56	18	-0.87	32	17	220	-0.77	7	43	11	15	270	0.95	37

[a] Parameters in Eq. 1.

lots after that incubation were much larger: 220 and 140 days, respectively.

6.2.3.2. *Biphasic decline of activity*

As described above, the decrease in the enzymic activity of Eupergit-C-immobilized cytosine deaminase was biphasic. So, it was assumed that the activity of the immobilized enzyme, A, at time t was the sum of activity of two kinds of immobilized enzyme residues with the activities of A_1 and A_2 at time zero, and with half-lives of T_1 and T_2, respectively, where the dimensions were units/gram of gel for A, A_1, and A_2; and days for t, T_1, and T_2. This relationship is represented by the following equation:

$$A = \frac{A_1}{2^{t/T_1}} + \frac{A_2}{2^{t/T_2}} \qquad \text{(Eq. 1)}$$

Through least-squares calculation for the curve of log$A$ *versus* t, suitable choices of the sets of parameters A_1, A_2, T_1, and T_2 were found; the results are given in the legend to figure 6-8 and shown in that figure by the dotted lines.

6.2.4. *Encapsulation of the immobilized enzyme*

6.2.4.1. *Enclosure in cellulose membrane*

The enzyme immobilized on Eupergit C, with 65 U of enzymic activity/g (wet), was enclosed in a tubular cellulose membrane for dialysis (with round-ended silicone caps on both ends of the tubing as described in figure 2-2). Each capsule contained 0.20 g of the gel (13 U of activity).

6.2.4.2. In vivo *stability of enzymic activity in the cellulose capsules*

One of the above enzyme capsules was implanted in four rats bearing the experimental brain tumours described in Chapter 2 and [57]; in three rats, the capsule was implanted under the skin, and in one rat, near the tumour. After 10 days, the rats were killed, the capsules were removed, cleaned with buffered saline, and assayed for enzymic activity with 1.0 mM cytosine (or 1.0 mM 5FC) as the substrate. The activity of the intact capsule was 0.014 ±

0.002 U for cytosine (0.013 ± 0.001 U for 5FC) in each capsule. The capsules then were cut with scissors, and the gel was removed and assayed for enzymic activity, which was 3.3 ± 0.1 U per capsule ($n=4$), corresponding to 25 % of the initial activity; this meant that the activity of the intact capsule was about 4 % of the activity inside the capsule.

6.2.4.3. *Entrapment in polyurethane*

The enzyme immobilized on Eupergit C, with 8.4 U of enzymic activity/g (wet), then was trapped in different amounts of polyurethane upon polymerization of the prepolymer. That is, Eupergit-C-immobilized enzyme was mixed with urethane prepolymer to make urethane-foam-like gels and cut with scissors into cubes about 2 mm to a side. The six gels made were assayed for enzymic activity (table 6-3), and the yield of apparent activity was 36 ± 8 %.

Table 6-3. Entrapment of Eupergit-C-immobilized cytosine deaminase upon polymerization of urethane prepolymer.

Cytosine deaminase immobilized on Eupergit C (8.4 U/g wet gel) with the indicated weight was suspended in 1.0 ml of saline and mixed with 0.5 g of PU-3, giving 1.5 g of polymer gel.

Gel no.	Immobilized enzyme used		Yield of enzymic activity	
	(g)	(U)	(U/g gel)	(%)
1 (△)	0.050	0.42	0.061	22
2 (▲)	0.050	0.42	0.12	42
3 (●)	0.10	0.84	0.22	40
4 (○)	0.10	0.84	0.26	47
5 (◆)	0.20	1.7	0.34	30
6 (□)	0.30	2.5	0.57	34

6.2.4.4. In vitro *stability of enzymic activity in polyurethane capsule*

Cubes weighing several tens of milligrams made of the above

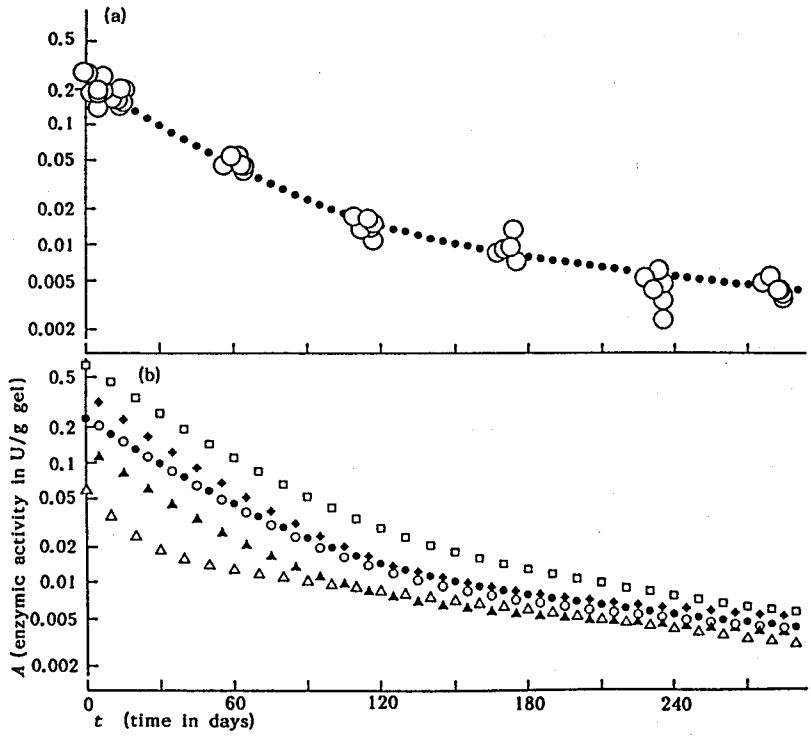

Figure 6-9. Stability at 37 °C of yeast cytosine deaminase immobilized on Eupergit C and entrapped in polyurethane.

(a) Gel no. 4 from table 6-3 was used.

(b) Only calculated results for the six gels are shown. Details are in the text.

Gel no.	Parameter				r	n
	A_1	T_1	A_2	T_2		
1 (△)	0.063	8.5	0.019	110	0.97	37
2 (▲)	0.12	24	0.0046	3,400[a]	0.99	39
	(0.12	19	0.01	160	0.98	39)[b]
3 (●)	0.21	22	0.018	140	0.99	39
4 (○)	0.23	22	0.012	190	0.99	39
5 (◆)	0.35	21	0.013	220	0.99	38
6 (□)	0.59	22	0.030	120	0.99	38

[a] Too large, so the calculation was done again with assumption of $T_2 = 160$, which the mean of T_2 for the five other gels (excluding gel no. 2). [b] Parameters in the parentheses are those obtained in the second calculation above.[a] r did not become much smaller. This is illustrated in the figure.

six gels were put into vials containing 1.0 ml of buffered saline and incubated at 37℃ for 9 months. At times, the remaining enzymic activity was assayed. Figure 6-9a shows the profile of decreases in the activity of gel no. 3 listed in table 6-3. This was a typical result; the activity decreased rapidly in the first 4 month, and slowly in the next 5 months. The half-life of activity was read from the linear graph and found to be 28 days ($r=-0.98$) during the first 4 months, but 99 days during the next 5 months. Equation 1 was also applied to the curve in figure 6-9a. Least-squares calculation led to similar results, which are listed in the legend to the figure together with those for the other five gels. The calculated results for the six gels are shown as rows of the symbols in fugure 6-9b.

6.3. DISCUSSION

The purpose of the study described in this chapter was to stabilize the yeast enzyme, which is very labile, for long-term use in the local cancer chemotherapy proposed in Chapter 2. The enzyme was stabilized through immobilization by various techniques. At first the beaded agarose, Sepharose 4B, was used. The gel was activated with CNBr, which is the best-known method to immobilize proteins on polysaccharide matrices. I used two improved techniques, which were developed to avoid frequent adjustment of the pH during the activation reaction needed with the usual methods; acetonitrile and an alkaline pH, or triethylamine and a neutral pH, were used. For greater simplicity, a commercial carrier, CNBr-activated Sepharose 4B, which is already activated and ready to use, was employed. Of these, only the triethylamine method gave both a good yield and good reproducibility. The poor yield with the acetonitrile method might be because cytosine deaminase itself is labile, especially at extremely alkaline pHs (as described in Chapter 5), where the coupling reaction occurs. The poor reproducibility might be because the alkali strength varies in different preparations. Such good results as were reported in the original article with this method [51] might not be obtained for alkali-sensitive proteins.

Eupergit A and C are commercial acrylic resins for the immobilization of proteins. Of these, Eupergit C gave both a good yield and good reproducibility, with a very easy

operation: the enzyme solution is merely poured over it. The merit of this carrier was its great capacity to immobilize enzyme activity, up to about 30 U per gram of completed resin. As described later, the resulting immobilized cytosine deaminase was stable for several weeks when incubated *in vitro* at 37℃. In another test (figure 6-7), after it was incubated for several months, during which period it lost much of the activity, it was more stable at the end of the test, the half-life of activity then being several months or more.

Formyl-Cellulofine was also a good carrier for cytosine deaminase. Its coupling capacity would be better when a purer enzyme is used, as in the Eupergit C experiments.

A general rule is that proteinous impurities in an enzyme solution diminish the coupling capacity of a carrier for the desired protein to be coupled. As can be seen in figure 6-3, with Eupergit C, the less the protein applied, the higher the yield; in each run, one of three enzyme solutions with various specific activities was used. When the yield was plotted as a function of the amount of the protein used for a fixed amount of the carrier (not shown), almost all points were on one curve, regardless of the specific activity of the enzyme solution used.

Urethane prepolymer is often used to immobilize biocatalysts such as microbial, plant, and animal cells, and cell organelles, immobilized enzyme gels, and microcapsules by entrapment [25]. The possibility of immobilization of soluble enzymes with such a prepolymer (Hypol EHP-3000) has been suggested elsewhere [77]. In related work here with this enzyme and PU-3, it was also true, but the enzymic activity of the resultant gel was not stable, as will be described below. Polyurethane gel has the useful characteristics of biocompatibility [69] and easy handling [25,26].

Urethane prepolymers polymerize through intermolecular condensation between isocyanate residues. Isocyanate residues on the prepolymer also react with amino groups, forming a urea linkage (without the liberation of carbon dioxide). So, any protein that has many free amino residues can be immobilized on polyurethane matrices through a condensation reaction between amino residues of the protein and isocyanate residues of the prepolymer *via* the urea linkage that is formed [77]. This means that proteins can be immobilized on gel matrices and not merely entrapped in the matrices being formed. This method would be useful for the immobilization of enzymes. Free cytosine deaminase retains only about half its initial activity after 30 min of incubation at 37℃, so the enzyme was in fact stabilized by use of a urethane polymer. However, the activity of the completed immobilized enzyme preparation was not as stable as with the

other immobilization techniques used here. The formation at times of linkages between the urethane prepolymer and impurities instead of the enzyme protein is suggested by the observation that the amount of the enzyme used and the yield changed reciprocally. The buffering effect of the support matrices against changes in the conditions of the micro-environment might contribute to the stabilization of enzyme activity, as is reported for gel entrapment with polyacrylamide, alginate, or κ-carrageenan [14].

Stabilization was the object of this chapter. Some preparations of the immobilized cytosine deaminase were examined in an accelerated stability test. They were selected according to the above results, being those that had been prepared with CNBr by the acetonitrile and triethylamine methods, and with Eupergit C, respectively. The test showed that all of the preparations were stable (figure 6-5). Further tests of *in vitro* stability at 37℃ for several weeks (figure 6-7) showed that the tendency for heat tolerance observed in this accelerated stability test was, after all, predictive of the actual long-term stability at 37℃. For stability of enzymic activity in the immobilized enzymes or in those entrapped further in capsules for still longer periods, the decline, fortunately, rapidly became less precipitous (within a few months). This suggested the presence of much stable factors among the many factors involved at the same time. In fact, factorization of the functional expression of the biphasic decline described above by practical computation showed that at least one factor was stable (figure 6-8, 6-9).

The apparent Michaelis constants, *Km*, for cytosine and 5FC of the immobilized cytosine deaminase, which were about 30 and 4 mM, respectively, with Eupergit C, were much larger than those of the free enzyme, which were about 3 and 1 mM, respectively (data not shown).

The enzyme capsules that I designed can be regarded as therapeutic bioreactors when embedded in a patient's body. Isolation of the enzyme protein from macromolecules in the body fluids is indispensable for prevention of allergic reactions. Here, urethane prepolymer was used to entrap the immobilized enzyme within gel blocks, as cellulose tubing was used to envelop the immobilized enzyme in Chapter 2. The advantage of the use of urethane is that the shape of the capsule can be custom-tailored [25], as follows. Polymer can be cast into any shape desired, and the completed gel can be reshaped with knife or scissors. The polymerization reaction occurs in mild conditions, and the process is simple.

Biocompatibility of the materials and of the completed bioreactor with the patient's body is the second necessity for therapy. Polyurethanes were such biocompatible materials

when used in experiments with rabbits and mice [69].

6.4. EXPERIMENTAL

Unless otherwise noted, all experiments were done as described in the previous chapters.

6.4.1. *Materials*

5FC was obtained from Daikin Industries (Osaka). Commercial CNBr-activated Sepharose 4B was obtained from Pharmacia. Eupergit A and C were manufactured by Röhm Pharma GmbH (Weiterstadt, F. R. G.). Formyl-Cellulofine was a product of the Chisso Corp. (Tokyo). The urethane prepolymers used, PU-3 and PU-6, were supplied by Toyo Tire & Rubber Co. (Osaka). Similar prepolymers are available from Bayer AG (Leverkusen, F.R.G.) and affiliated companies as Desmodur T-80 [42] and from W. R. Grace & Co. (Columbia, Md.) as Hypol EHP-3000 [77].

6.4.2. *Enzyme assay*

Enzyme activity was measured by dual-wavelength spectrophotometry as described in Chapter 2. The enzymic activity in the immobilized enzyme gels was expressed as the apparent activity found by the assay of cytosine and uracil in the assay solution (with the immobilized enzyme gel removed) in the same way as above for the activity of the free enzyme. The activity of the enzyme immobilized in polyurethane (PU) was expressed as the apparent activity, as follows. The PU gel was cut into cubes several millimetres to a side with scissors, put into a screw-capped test tube, and then incubated with several changes every few hours of 1.0 ml of 1.0 mM cytosine (or 5FC) in phosphate-buffered saline. The solutions removed were analysed spectrophotometrically for changes in the substrate and product concentrations during incubation, which concentrations converged to certain values over time. The apparent enzymic activity in the PU gel was calculated from these values, as described previously for the enzyme bags.

6.4.3. *Preparation of the enzyme*

Enzyme was usually prepared as before, using 0.5 ~ 1.5 kg of compressed yeast for every preparation. Typical steps included autolysis of the yeast, salting-out with ammonium sulphate, and anion-exchange and hydrophobic chromatography. A final fraction from 1.5 kg of yeast, for example, contained 200 U of activity (6.2% yield) with a specific activity of about 110 U/mg of protein, which was 42% that of the purest form of the enzyme (250 U/mg) that had been obtained in Chapter 5.

6.4.4. *Immobilization techniques*

6.4.4.1. *Activation of Sepharose 4B with CNBr*

Sepharose 4B was activated with CNBr by the following three methods.

Acetonitrile method [51]. The acetonitrile method was used in preparation of affinity adsorbents in the studies of purification of the *E. coli* enzyme (Chapter 4). The standard conditions for activation were as follows: 0.50 ml of the CNBr /acetonitrile solution (100 g/50 ml) was used per 5 g of wet Sepharose 4B suspended in 20 ml of 1 M sodium carbonate (at about pH 10), with activation for 1 min. After activation, Sepharose 4B was used immediately. Coupling was done for 2 days so that unreacted active groups would be masked. The conditions chosen here as being optimum, although the reproducibility of the activation reaction was poor, were the use of 50 μl of the CNBr solution per gram of gel and reaction for 1 min.

Triethylamine method [44]. The triethylamine method was as follows: 2.5 ml each of 1 M CNBr and 1.5 M triethylamine stock solutions in 60% acetone were used for 10 g of wet Sepharose 4B. The amount of cyanate ester that formed on the gel was assayed before the gel was used for immobilization. According to the results of preliminary experiments, the gel was activated more by larger amounts of CNBr, and the amount described above was enough; over 20 μmol of the ester was formed.

Ready-to-use carrier. A pre-activated carrier is commercially available as CNBr-activated Sepharose 4B. The dry resin was wetted and washed before use as described by the manufacturer. Coupling was done overnight in a refrigerator. To block unreacted active residues, the gel was removed with filter paper under reduced pressure, transferred into several volumes of 0.2 M Tris-HCl buffer (pH 7.0), left in the cold for 2 days, and then thoroughly washed with the phosphate buffer with use of filter paper.

6.4.4.2. *Eupergit A and C*

Eupergit A and C were used as indicated by the manufacturer, except that Eupergit A was used dry. The buffers used were of neutral pH. Immobilization involved the mixing of the resins with the enzyme solution. The mixtures were then left in a cool place for a few hours or days. The amount

of enzyme solution used was 4 ml per gram of dry resins.

6.4.4.3. *Formyl-Cellulofine*

The coupling buffer for Formyl-Cellulofine was 0.2 M KH_2PO_4/0.1 M NaCl, adjusted to pH 7.0 with NaOH. The wet carrier gel was put on a sintered-glass filter, washed thoroughly with water under reduced pressure, and transferred to a beaker containing 20 ml of the coupling buffer per 1.0 g of gel. After being stirred for about 30 min, the gel was collected with the filter and transferred to a small beaker; 2.0 ml of the coupling buffer containing cytosine deaminase to be coupled was added and the mixture was gently shaken for 30 min. Coupling was started by the addition of 7 mg of $NaCNBH_3$, and the mixture was shaken gently overnight before being washed with 0.2 M Tris-HCl buffer (pH 7.0) on filter paper. Unreacted residues were blocked with 2 ml of the same buffer containing 5 mg of $NaCNBH_3$, with overnight shaking. The gel was washed thoroughly with phosphate-buffered saline over the filter and stored in a refrigerator until use.

6.4.4.4. *Urethane polymerization*

The urethane prepolymers used were those developed by Fukui and coworkers [25,26]. The prepolymer was polymerized as follows. Usually a hydrophobic prepolymer, PU-3, was used. PU-6 is hydrophilic. The prepolymer was weighed into a vial or a beaker; for example, 1 g was put into a 100-ml vial (35 mmϕ) to obtain a polymer layer about 5 mm thick. If sterilization was necessary, the prepolymer could be autoclaved in a vial with a Teflon-lined screw cap or heated at 110℃ in an oven in a beaker with aluminum foil over it. The prepolymer was heated to melt it if it was solidified, and then cooled to a moderate temperature. To the prepolymer (1 g, in this instance) was added 2 ml of the enzyme solution, followed by mixing with a microspatula at room temperature. After foaming began, arising from the condensation reaction, which generated CO_2, mixing was continued for several minutes until gellation started, and then the mixture was placed in a refrigerator and stored for a few hours for polymerization to be completed. The polymer, which was like urethane foam, was removed from the vial and cut into pieces with scissors. The gel was then weighed. If necessary, the gel was degassed in a beaker containing buffered saline under reduced pressure in a vacuum bell and washed with several changes of the buffered saline, after which it weighed a few tens of percent more and was clear, being free from gas.

6.4.5. *Sterile techniques*

The immobilized enzyme (except that with polyurethane) was sterilized with buffered saline containing 0.1% NaN_3, 0.1 % Osvan, or 1 mM $HgCl_2$ for 1 hr on a clean bench, and then washed thoroughly with the buffered saline on a sintered-glass filter. For polyurethane, sterilization was done before polymerization as follows: the prepolymer was sterilized as described before in section 6.4.4.4 and then the enzyme solution was sterilized by microfiltration (0.22-μm pores). Polymerization was done on a clean bench.

6.4.6. *Least-squares calculation*

Least-squares calculation was done on a personal computer with application of the conjugate descent method of Davidon with use of iterative calculation, as modified by Fletcher and Powell [24], which calculation had been fitted for general uses in least-squares calculation (not shown).

A was a function of the variables A_1, A_2, T_1, and T_2 as represented by equation 1 in the results section (6.2.3.2). Starting at the proper combination of values of the variables A (*i.e.*, A_{obs}) at every point (time) of observation (*i.e.*, t) was calculated directly from the equation. The sum of the squares of distances of $\log A_{obs}$ from $\log A$ on the curve was used as the assessment function. Variables were changed during computation so as to minimize the function by a programme (figure 6-9) that was fitted for this case from the generalized one described above.

```
100 '   ********************************************************************
    '   *                                                                  *
110 '   *          Davidon-Fletcher-Powell Least-squares Method            *
    '   *                                                                  *
    '   ********************************************************************
120 '
130 '
200     COLOR 4 : CLS
210     PRINT : PRINT "WELCOME to Davidon-Fletcher-Powell's Least Squares !"
220     GOTO 10000
230 '
240 '
1000 '  ********************************************************************
    '   *  Main Routines                                                   *
    '   ********************************************************************
1010 '
1100 '  **********
1110 '    *EQTN
1120 '  ┌──────────────────────────────────────────────────────────────────┐
1130 '  │    ACT = X(1) / 2^( DTE(I)/X(2) ) + X(3) / 2^( DTE(I)/X(4) )     │
1140    └──────────────────────────────────────────────────────────────────┘
1150 RETURN
1160 '
1200 '  **********
1210 '    *FCL
1220 '
1230 FX=0 : MN=MN+1
1240 FOR I=1 TO N                                    GOSUB *EQTN
1250   FX = FX + ( LOG (ACT) - LOG (DTA(I)) )^2
1270 NEXT
1280 RETURN
1290 '
1300 '
2000 ' ****************** START ***********************************************
2010 '    *START
2020 '
2030 EQ=.01:EU=1+EQ:EL=1-EQ
2040                                                 GOSUB *FCL : FI=FX
2050                                                 GOSUB *COR : GOSUB *SD
2060 '
2070 FOR I=1 TO N1
     FOR J=1 TO N1
```

Figure 6-10. BASIC programme of least-squares calculation on a personal computer for equation 1.

Data and tentative parameters are input in the programme after line 20000 (*i.e.*, the data input subroutines).

The printer is regulated, in this case, under the ESC/P mode (Epson Corp.(C)).

```
 H(I,J)=0
NEXT J,I
FOR J=1 TO N1
 XS=X(J)
 X(J)=XS*(1+EQ) :                                  GOSUB *FCL :  FF=FX
 X(J)=XS*(1-EQ) :                                  GOSUB *FCL :  FR=FX
G0(J)=(FF-FR)/(2*EQ*XS)
 X(J)=XS
IF FF+FR-2*FI<=0 THEN H(J,J)=1:                    GOTO  2180
 H(J,J)=(EQ*XS)^2/(FF+FR-2*FI)
NEXT
'
'
' ****************** MAIN ROUTINE ***************************************
'     *MAIN
'
A1=X(1) : T1=X(2) : A2=X(3) : T2=X(4)
FOR I=1 TO N1
 D(I)=0:BN=0
 FOR J=1 TO N1
  D(I)=D(I)-H(I,J)*G0(J)
 NEXT J
 BN=BN+D(I)*D(I)
NEXT I
BN=SQR(BN)
'
GD=0
FOR J=1 TO N1
 D(J)=D(J)/BN
 GD=GD+G0(J)*D(J)
 XX(J)=X(J)
NEXT J
'
IF GD>=0                                           GOTO  *RESTART
'
'
'                *** Searching Minimum Point on a Line ***
'
RL=10
A=0:R1=GD:FO=FI
BB=0:FMIN=FI
'
' **********
'     *LOOP
'
B=A-2*FO/R1
IF B>A+RL THEN B=A+RL
 FOR I=1 TO N1
X(I)=XX(I)+B*D(I)
NEXT
'                                                  GOSUB *FCL : FM=FX
FOR I=1 TO N1
 X(I)=XX(I)+(B*EU)*D(I)
NEXT
'                                                  GOSUB *FCL : R2=(FX-FM)/B/EQ
IF R2<0 THEN R1=R2:A=B*EU:FO=FX:                   GOTO  *LOOP
'
LOOP=0
'
' **********
'     *SLP
'
S=3*(FO-FM)/(A-B)
Z=S+R1+R2
W=SGN(B-A)*SQR(Z*Z-R1*R2)
RN=A+(B-A)*(W-R1-Z)/(R2-R1+2*W)
FOR I=1 TO N1
X(I)=XX(I)+RN*D(I)
NEXT
'                                                  GOSUB *FCL :  F=FX
IF (F<FM)*(F<FO) THEN *CNTN                        THEN  *CNTN
IF LOOP>4
'
FOR I=1 TO N1
 X(I)=XX(I)+(RN*EU)*D(I)
NEXT
'                                                  GOSUB *FCL :  R=(FX-F)/RN/EQ
IF (R*R1)>0 THEN A=RN:R1=R:FO=FX :                 GOTO  5200
B=RN:R2=R:FM=FX
LOOP=LOOP+1 :                                      GOTO  *SLP
'
' **********
'     *CNTN
'
IS=IS+1
'                                                  GOSUB *COR :  GOSUB *SD
FR = F/FI
IF FR =< .999995 THEN 6200
IF FR =< 1       THEN LPRINT                       THEN  *CVG
IF FR > 1
       Error in parameter conversion !  ---   Restart !  ---":
                                                   GOTO  *RESTART
'
FI=F
'
'
' ****************** MATRIX CONVERSION ***********************************
'
FOR J=1 TO N1
 XS=X(J)
 X(J)=XS*EU :                                      GOSUB *FCL : FF=FX
 X(J)=XS*EL :                                      GOSUB *FCL : FR=FX
 X(J)=XS
G1(J)=(FF-FR)/(2*BQ*XS)
 Y(J)=G1(J)-G0(J)
NEXT
'
FOR I=1 TO N1
 HY(I)=0:YH(I)=0
  FOR J=1 TO N1
   HY(I)=HY(I)+H(I,J)*Y(J)
   YH(I)=YH(I)+Y(J)*H(J,I)
NEXT J,I
'
YHY=0:DS=0
FOR J=1 TO N1
 DG=DG+D(J)*Y(J)
 YHY=YHY+Y(J)*HY(J)
NEXT
'
FOR I=1 TO N1
 FOR J=1 TO N1
 DD(I,J)=D(I)*D(J)
 HYYH(I,J)=HY(I)*YH(J)
NEXT J,I
'
FOR I=1 TO N1
 FOR J=1 TO N1
NEXT J,I
FOR J=1 TO N1
 G0(J)=G1(J)
NEXT
'
                                                   GOTO  *MAIN
'
'
' ****************** CONVERGENCE *****************************************
' **********
'     *CVG
'
A$="                    Convergence !
PRINT A$ : LPRINT CHR$(27);"G"; A$; CHR$(27); "H"
INPUT "Restart --- / n(o    "; R$
IF R$="                                            THEN  *RESTART
                                                   GOTO  *RSLT
'
'
' ********** CORRELATION COEFFICIENT AND STANDARD DEVIATION **************
' **********
'     *COR
'    (r, correlation coefficient)
F1=0 : F2=0 : F3=0 : F4=0 : F5=0
FOR I=1 TO N                                       GOSUB *EQTN
    YI = LOG (ACT)
    Y2 = LOG (DTA(I))
    F1 = F1 + YI
    F2 = F2 + YI*Y2
    F3 = F3 + YI^2
    F4 = F4 + Y2
    F5 = F5 + Y2^2
NEXT
'
    COR = ( F2 - F1*F4 / N ) / ( F3 - F1^2 / N )^.5 / ( F5 - F4^2 / N )^.5
'
RETURN
'
' **********
'     *SD
'
SDE = SQR ( FX / (N-1) ) : SD = SDE / 2.3026 : EX = 10^SD
'
' ***************** DISPLAY AND PRINT OUT OF THE RESULTS ******************
'                                                                          "
    F$ =
#### ###.###### .##### .### #.### ###.#### ###.##### ###.#### #####.####"
  PRINT USING F$; IS, MN, FX, COR, SD, EX, X(1); X(2); X(3); X(4)
 LPRINT USING F$; IS, MN, FX, COR, SD, EX, X(1); X(2); X(3); X(4)
RETURN
'
'
' ********************************************************************
'  Pre- & Sub-Routines                                              *
' ********************************************************************
'
' ********************************************************************
' Printer Setting
'
LPRINT CHR$(27); "m"; CHR$(0); CHR$(27); "@" :  REM◄ STD mode
LPRINT CHR$(27); "C"; CHR$(72); CHR$(27); "N"; CHR$(12); :
                      REM◄ page formatting (12") & perforation skip (2")
LPRINT CHR$(27); "P"; CHR$(15); CHR$(27); "O"; :
                      REM◄ VERT & HRZ formatting (1/2 pica; 1/8")
LPRINT CHR$(27); "l"; CHR$(6); CHR$(27); "Q"; CHR$(96); :
                      REM◄ Left and right margin
'
'
' ********************************************************************
' Title and Initial Data Printed
'
PRINT "? Date XX/YY/ZZ  +/ Time,etc. to be printed -- ["; DATE$; :
INPUT "] / else "" D$ : IF D$="" THEN D$=DATE$
LPRINT CHR$(27);"GDFP";,,,, D$; CHR$(27); "H" :  REM◄ Title printed
LPRINT
'
'
' ********************************************************************
' Error Trap
'
ON ERROR                                           GOTO  12050
                                                   GOTO "13030
   PRINT      "Error"; ERR; "! on"; ERL
  LPRINT "       Error"; ERR; "! on"; ERL; " --- ' Restart ! --- "
RESUME                                             *RESTART
'
   PRINT "Error"; ERR; "! on"; ERL ; "    ";
'
'
' ********************************************************************
'   Stop (Help)
'
ON STOP                                            GOSUB *PARAMETERS
STOP ON
KEY 1, "*RSLT"+CHR$(13): KEY 2, "*RESTART"+CHR$(13):
KEY 7, "*PROGRAM "+CHR$(13)
                                                   GOTO  15010
'
'
' ********************************************************************
' Definitions of the other * labels
'
' **********
' *PARAMETERS
'
STOP OFF : PRINT "STOP !"; :
  LPRINT "       Stop ! ---   Restart ! ---   "
PRINT "       Hit f-3 & f-2 keys for restart !"
STOP
'
'
' ****************** RESTART *********************************************
'
' **********
' *RESTART
'
STOP ON
PRINT "RESTART !  ";
INPUT "Enter the new parameters ! ", IS, MN, X(1), X(2), X(3), X(4)
MN=MN-1
                                                   GOTO  *START
'
'
' ****************** EXIT TO SYSTEM **************************************
'
' **********
' *PROGRAM
'
KEY 1, "load "+CHR$(&H22): KEY 2, "auto ": KEY 7, "key "
END
'
'
' ****************** DIMENSIONS ******************************************
'
DIM DTE(128), DTA(128)
DIM X(20),H(20,20),G0(20),G1(20),D(20),Y(20)
DIM HY(20),YH(20),HG(20),DD(20,20),HYYH(20,20)
'
'
' ****************** NO. OF VARYING PARAMETERS ***************************
'
PRINT "? No. of the varying parameters --- 4 / else ?" :" ;
PRINT "      ( Enter 100 for manual operation. )       " ; :
INPUT N1 : IF N1=0 THEN N1=4  ELSE IF N1>99 THEN N1=0
'
```

Figure 6-10 (continued).

```
20000 ' ****************** DATA ENTRY ********************************************
20010 '
20020 '
        Use editor !. Data is entered from the other ASCII file by merging
20030 '  before run of this programme.
20040 '
20990 '
        Enter the name/number/date of the data to be entered !
21000     A$="Data 84/-6/21; corre. 6/9/18
21010 READ X(1), X(2), X(3), X(4)
21020 X(I)=XX(I)
21030 '
21100  PRINT A$
21110 LPRINT CHR$(27); "G"; A$; CHR$(27); "H"
21120 '
21990 '
        Enter the temporary initial A1, T1, A2, and T2 values !
22000     DATA   20,   10,   10,   200
22010 A1=X(1) : T1=X(2) : A2=X(3) : T2=X(4)
22020 '
22990 '
        Enter date and data ! --- DTE, DTA,   DTE, DTA,  .....
23000  DATA   0,  29.8 ;    0,  29.1 ;    1,  25.3 ;    1,  24.3 ;    2,  27.9 ;
              2,  26.6 ;    4,  25.3 ;    4,  22   ;    6,  20.1 ;    6,  19.8 ;
              8,  22.3 ;    8,  19.8 ;    8,  18.5 ;   11,  17.6 ;   14,  21.5
23010  DATA  14,  15.9 ;   18,  18   ;   18,  17   ;   21,  21.2 ;   21,  17.3 ;
             97,  10.6 ;   97,   7   ;  132,  11.2 ;  132,   8.28;  228,   6.48;
            228,   6.07
23020 '
23030 '
23040 '
23050 '
23990 '
        Enter the number of the data points !
24000     N = 26
24010 '
24020 '
25000 ' ****************** DATA IN ***********************************************
25010 '
25020 FOR I=1 TO N
25030     READ DTE(I),  DTA(I)
25040 NEXT
25050 INPUT "? Print data points --- / n(o                    "; PR$ :
      IF PR$<>"n"                                        THEN 26000
25060 LPRINT "     ( Dte and Dta ( log Dta ) of the";
25070 LPRINT CHR$(27); "G"; N; CHR$(27); "Hpoints ]:"
25080 LPRINT CHR$(27); "I"; CHR$(21);
25090 F$="###  ###.### (##.### )
25100 FOR I=1 TO N
25110  LPRINT USING F$;  DTE(I), DTA(I), LOG ( DTA(I) ) / 2.3026;
25120 NEXT : LPRINT
25130 '
25140 '
26000 LPRINT CHR$(27); "1"; CHR$(16)
26010 '
26020 '
27000   A$ =                                                                  "
========================================================================================
Step  Calc  Fx       r      s.d.  exp    A(1)       T(1)       A(2)       T(2)"      "
27010   B$ =                                                                          "
----------------------------------------------------------------------------------------
"
```

```
27020   PRINT A$ : PRINT B$;
27030 : LPRINT A$ : LPRINT B$
27040 '
28000 '                                                GOTO  *START
28010 '
28020 '
30000 ' ****************** PRINT OUT THE RESULTS IN A TABLE ********************
31010 '
31020 ' **********
31030 ' *RSLT
31040 '
31050 BEEP : BEEP : BEEP : INPUT "? Form feed --- / n(o "; FD$ :
      IF FD$="n" THEN LPRINT  ELSE LPRINT CHR$(12)
31060 '
31070 INPUT "? Parameters to be rounded to fewer decimals --- / n(o ", PM$ :
      IF PM$<>"n" THEN
      INPUT "Then, enter A(1), T(1), A(2), T(2) !      ", A1, T1, A2, T2
31080 X(1)=A1 : X(2)=T1 : X(3)=A2 : X(4)=T2
31090 '                                                GOSUB *COR
31100 '
31110 LPRINT : LPRINT "Curve:"; CHR$(27); "G";
        Act(dte) ="; A1; "/ 2 ^ ( dte /"; T1; ")+";
                 A2; "/ 2 ^ ( dte /"; T2; ")"; CHR$(27); "H" ;
31120 LPRINT USING "      r = #.####"; COR
31130 '
31140 LPRINT : LPRINT                                                              "
========================================================================================
31150 LPRINT                                                                       "
Days  +0                                  +1  +2  +3  +4  +5  +6  +7  +8  +9"
31160 LPRINT                                                                       "
---------------------------------- --- --- --- --- --- --- --- --- ---
31170 LPRINT                                                                       "
Act]  (log) Act2    (log) Act    (log)  log log log log log log log log log
31180 LPRINT
----------------------------------------------------------------------------------------
31190 '
32000 F$ =                                                                         "
 ###  ###.# ##.## ###.## ##.## ###.# ##.### "
32010 FOR I=0 TO 290 STEP 10
32020 '
32030   ACT1 = A1 / 2^(I/T1) :  ACT2 = A2 / 2^(I/T2) :  ACT = ACT1 + ACT2 :
        L1 = LOG (ACT1) / 2.3026 :  L2 = LOG (ACT2) / 2.3026 :
        L  = LOG (ACT)  / 2.3026
32040 '
32050   LPRINT USING F$; I, ACT1, L1, ACT2, L2, ACT, L ;
32060   FOR J=1 TO 9
32070    ACT = A1 / 2^((I+J)/T1) + A2 / 2^((I+J)/T2) : L = LOG (ACT) / 2.3026
32080    L=L-FIX(L)
32090    L=L*1000 :
         IF L>=999.5 THEN L=0
32100    LPRINT USING "  ###"; L;
32110   NEXT J : LPRINT
32120 NEXT I : LPRINT
32130 '
32140 '
32150 INPUT "? One more curve with the parameters changed --- /n(o ", MC$:
      IF MC$<>"n" THEN                                 *RSLT
32160 '
32170 '
40000 ' ****************** END ***************************************************
40010 '
40020 LPRINT CHR$(27); "1"; CHR$(16); :                  REM◢ Left margin
40030 '
40040 PRINT : PRINT "END !  BYE !" : PRINT
40050 GOTO *PROGRAM
40060 END
40070 ' ************************************************************************
```

Figure 6-10 (continued).

CHAPTER 7

Conclusions of this series of studies of microbial cytosine deaminases and their use in a new kind of cancer chemotherapy

In this chapter will be described the conclusions of this series of studies. I studied the use of microbial enzymes for treatment of cancer. I described the background of the studies in the introductory part (Chapter 1). 5FU has antitumoural and a strong, broad-range antimicrobial spectrum, but is toxic and causes side effects. 5FC has no antitumoural or bacteriostatic activity, and little clinical toxicity.

My approach to a solution to the problem of side effects was a drug-delivery system for the controlled release of 5FU. I wished to use 5FC as the depot form of 5FU in the treatment of cancer. The mode of action of 5FC in the proposed programme would be as follows. Cytosine deaminase would be injected or implanted near the tumour in a cancer patient; 5FC would be given periodically to the patient by mouth; after it arrived at the enzyme capsule *via* the blood, it would be converted to 5FU at the site by deamination by the action of the enzyme; 5FU would then act on the tumour. The local level of 5FU would be semi-constant. Cytosine is not metabolized in mammals, which have no cytosine deaminase. Cytosine deaminase is unusual in that it is found only in microbes.

7.1. *5FC-deaminating activity of cytosine deaminase*

To start with, an enzyme that deaminated 5FC to 5FU was searched for. A cell extract of *Escherichia coli* was found to have a cytosine deaminase that can deaminate 5FC to 5FU. The enzyme was very stable to heat.

7.2. *Possible cancer chemotherapy with 5FC and cytosine deaminase*

I designed enzyme capsules containing cytosine deaminase,

made with cellulose semipermeable membranes to avoid reaction of the enzyme protein with macromolecules in the body. The enzyme was partially purified and aseptically encapsulated in semipermeable cellulose tubes.

Preliminary experiments with the enzyme capsules and with experimental brain tumours in rats were carried out in collaboration with neurosurgeons at Kansai Medical University, Moriguchi, Osaka. Encouraged by the results obtained in the above experiments, I started this study.

An enzyme capsule was incubated in sterile saline at 37℃. After 16 days, it had some enzymic activity. Further experiments were done with experimental brain tumours in rats. Capsules were implanted under the skin of the rats near the tumours. After one month, the capsules were found to contain some activity (biological half-life of 10 days). These findings suggested that the capsules might be used for the chemotherapy of cancer.

7.3. *Cytosine deaminase from* Escherichia coli

A large amount of this enzyme will be necessary if it is to be used in therapy, and it will probably need to be fairly pure. Preliminary experiments showed that the amount of the enzyme protein in the cell extract was very small and that purification on the scale of about a thousand times was necessary.

Cultural conditions for enzyme production were investigated. Citric acid was a suitable carbon source. The pH of the culture increased during cultivation, and growth and enzyme activity increased when the pH was controlled so as not to exceed a certain value. Control of the culture pH at around 8.5 with citric acid was most effective for enzyme production. So, a pH-stat was constructed. Citric acid was added to the culture by the pH-stat and then large-scale production of the enzyme was possible. *E. coli* was cultured in tanks under the optimum conditions established, and the enzyme was purified 1,200-fold from the extract of the cells, to homogeneity, by the usual procedures plus column chromatography by use of affinity adsorbents prepared for this purpose. The activity in the cell extract was still stable after the purification; achieving this thermostable nature was the main objective of the study in Chapter 4. Enzyme activity was optimum at around pH 9. The enzyme was active at the region of physiological pH, which is promising for the clinical use of this enzyme.

7.4. *Cytosine deaminase from bakers' yeast*

Cytosine deaminase from *E. coli* could not yet be used in the proposed method for the local chemotherapy of cancer. One reason is that, this cytosine deaminase was obtained from *E. coli* only in low yield when highly purified and it is difficult to culture the bacteria on a large scale in order to obtain enough activity. Second, because of possible pyrogenic reactions, it is preferable to use a highly purified preparation of the enzyme or else to use safer sources than bacteria.

Cytosine deaminase can, however, be obtained in large quantities from commercial compressed bakers' yeast, and the organism seems to be relatively safe. Yeast cytosine deaminase deaminates 5FC.

This enzyme was extracted from commercial compressed yeast and purified 6,800-fold to an almost homogeneous state. The enzyme activity was more than 200 U/mg of protein, which was the purest preparation from bakers' yeast reported so far.

The enzyme activity was as labile as when the enzyme was a crude or partially purified preparation. This would cause difficulties if it were used in long-term use in the local cancer chemotherapy proposed.

7.5. *Stabilization of cytosine deaminase from bakers' yeast by immobilization*

The yeast cytosine deaminase was stabilized through immobilization. The enzyme was immobilized on many carriers by various relatively simple techniques, and tested for stability at 37℃. Some preparations of the immobilized cytosine deaminase were examined in an accelerated stability test before the long-term tests at 37℃.

When the enzyme was immobilized on Eupergit C, a commercial epoxy-acrylic resin, the highest yield of apparent enzymic activity was about 80 % of the starting activity. The activity decayed exponentially at 37℃ over a few weeks, the biological half-life being about 10 days. Other commercial adsorbents also resulted in good stability but yields were lower, being 20~67 and 21% for Formyl-Cellulofine and CNBr-activated Sepharose 4B, respectively, with half-lives of 15

and 24 days.

7.6. *Enzyme capsules in therapy*

The immobilized enzyme might be encapsulated for therapy, in cellulose membrane or with urethane prepolymer, which are easily handled; in addition, polyurethane has biocompatibility with animal tissues. The Eupergit-C-immobilized enzyme was further encapsulated in cellulose tubing for dialysis or else entrapped with urethane prepolymer. Such enzyme capsules were stable; the polyurethane capsules had a half-life of activity of about 5 months when incubated for 4 months at 37℃. The intact enzyme capsules had 4% of the original activity of the enzyme immobilized inside the capsules.

The enzyme capsules I designed can be regarded as therapeutic bioreactors when embedded in a patient's body. Prevention of allergic reactions was brought about by entrapment of the immobilized enzyme within gel blocks of the polyurethane to isolate the enzyme protein from macromolecules in the body fluids. Urethane prepolymers as the materials and the polyurethane as the resulting bioreactor are compatible with the patient's body.

The experiments here were carried out to find whether requirements for the proposed cancer chemotherapy were fulfilled. First, 5FC must be delivered to the diseased area: this should occur, as 5FC readily passes the blood-brain barrier in the chemotherapy of fungal diseases. Second, the enzyme capsule *in situ* must deaminate 5FC to form 5FU: this was found to be true. Third, the enzyme must have long-term stability in the body: this was attained here, as described above for Eupergit-C-immobilized cytosine deaminase entrapped in polyurethane.

In therapy, the enzyme capsule could be implanted during the surgical removal of most of the tumour. This method might make it possible to use 5FC as postoperative therapy. Such therapy would be advantageous, especially in the brain where total resection of the tumour is not always possible. Because 5FC can be taken by mouth, this method would be convenient for long-term care. This is not true of current modes of therapy that are usually restricted to hospitalized patients.

The results of the studies suggested that the method of use could become a new kind of chemotherapy, and, at the same time, a new kind of enzyme application. Use of enzymes

is a well-established technology. Immobilization of enzymes to increase stability of enzymes has been done for some time. However, when new materials obtained from the use of "new technology" were used to immobilize an enzyme here, the enzyme could be made into a stable, safe, biocompatible enzyme capsule. The results might be called a fruit of the new biotechnology. The concerted effects coming from the use of these "old" and "new" technologies together are very promising.

7.7. *Miscellaneous*

In the course of these studies, I also dealt with many related problems as follows.

7.7.1. *Visual agar-plate assay for deaminases with use of a pH indicator*

Before starting the large-scale purification of cytosine deaminase, I devised an easy, simple, rapid method for the detection and assay of cytosine deaminase.

A paper-disk-agar-plate method with use of a pH indicator was developed for the assay of adenosine deaminase. Then, this method was used with cytosine deaminase, the subject of this series of studies. The method was used to detect and estimate amounts of specific inhibitors of adenosine deaminase.

This proposed method is also advantageous when used to detect activity in a large number of samples such as fractions from column chromatography of deaminases or their inhibitors. After several hours of practice, activity can be detected at a glance.

The value of this method for detection of inhibitors is enhanced by its specificity for specific inhibitors only, and the method should be applicable for screening for inhibitors of adenosine deaminase.

7.7.2. *Affinity adsorbents for the purification of* E. coli *cytosine deaminase*

For efficient purification, use of affinity chromatography seemed to be suitable. Many classes of pyrimidine compounds were immobilized on Sepharose 4B by the construction of

various spacers by cyanogen bromide activation of the carrier. Crude enzyme solutions were studied by adsorption and desorption chromatography with columns of the 68 kinds of gels thus obtained. The effects of the techniques employed were discussed above as well as the effects of the lengths or the hydrophobicity of the spacers. Some gels were effective in purification.

7.7.3. *Comparative studies of cytosine deaminases*

I highly purified cytosine deaminases from *Escherichia coli* and bakers' yeast (*Saccharomyces cerevisiae*) for use in these studies. Although the enzymes of many microorganisms including both of these organisms had already been studied, these two enzymes were studied in detail because they were newly purified to a high degree.

This enzyme from *E. coli* was thermostable. The enzyme from bakers' yeast was unstable to heat, with a biological half-life of about 0.5 hr at 37℃ (Chapter 5). They had molecular weights of about 200,000 and 40,000, respectively. 5FC, 5-methylcytosine, and creatinine were other substrates for both enzymes. Inhibitor studies suggested that they were SH-enzymes. About half of all bacterial cytosine deaminases are thermostable, and the others are unstable, as are the fungal enzymes.

The molecular weights of the fungal cytosine deaminases are 30,000～40,000; very different from those of the bacterial enzymes, which are 200,000～600,000.

5MC and 5FC were deaminated by cytosine deaminases from both fungi and some bacteria, to different extents; the conversion of 5FC to 5FU by cytosine deaminase in fungi explains the antifungal activity of 5FC. Most cytosine deaminases, with rare exceptions, deaminate creatinine.

Cytosine deaminase from *E. coli* had been believed not to deaminate 5-substituted cytosines. In fact, cytosine deaminases from two derivative strains of *E. coli* did not deaminate 5FC or 5MC at all, and the activities toward 5MC and 5FC in comparison to the cytosine values varied from strain to strain in some stock strains of *E. coli*.

Bacterial cytosine deaminases have been classified according to substrate specificity and heat stability into the *Serratia* type, which is cytosine-specific and stable, and the *Pseudomonas* type, which is non-specific and unstable. The *Escherichia* enzyme had been classified into the *Serratia* type, but I here characterize it as a third type, called the *Escherichia* type, because of its reactivity toward 5MC and 5FC being unlike that of the *Serratia* type.

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